Detection and initial characterization of synovial lining fragments in synovial fluid

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Objective. Free fragments of synovium have occasionally been seen in synovial fluid but have not been studied systematically. We wished to establish a method for the reliable detection of these fragments in joint and bursa effusions and begin to characterize them by histochemical and immunohistochemical methods.

Methods. Cell smears, wet drop preparations and cytopspins were prepared from 39 consecutive joint and bursa effusions. Paraffin cell blocks were prepared from a subset. Analysis encompassed standard and polarized light microscopy, histochemistry, immunohistochemistry and transmission electron microscopy (EM). Synovial biopsy tissue from one different patient was examined for comparison.

Results. Tissue fragments were not seen in Wright-stained cell smears and only rarely in wet drop preparations. In contrast, variously sized fragments with the histological appearance of hyperplastic synovial lining were detected in ethanol-fixed, haematoxylin/eosin-stained cytopspins from bursitis and all arthropathies studied [17/24 (71%) of non-inflammatory and 12/15 (80%) of inflammatory specimens]. Immunostaining revealed CD68 expression in a subset of cells in a pattern characteristic of hyperplastic synovial lining. Juxtaposed cells with morphological features of macrophage-like and fibroblast-like synoviocytes were seen by EM.

Conclusions. Synovial lining fragments can be detected in effusions from diverse arthropathies and bursitis. They maintain important properties of the synovial lining and can be analysed by immunohistochemistry. They may afford the opportunity to study a relatively pure preparation of synovial lining cells without the need for cell culture, and to evaluate their possible role in augmenting or perpetuating synovitis or joint damage.

Key words: CD68, Rice bodies, Synovial lining, Synovial fluid, Synovial fluid analysis, Synovium.

Inflammatory cells and individual synovial lining cells are easily detected in synovial fluid (SF) and have been the subject of numerous studies. Recent work has focused on cultured SF fibroblasts to study the synovial lining [1, 2], but artefacts resulting from ex vivo propagation may be confounding factors. Intact synovial lining tissue, on the other hand, needs to be obtained by more invasive procedures, i.e. arthroscopy, closed needle biopsy or open biopsy. It would therefore be desirable to obtain synovial lining tissue by additional, less invasive methods, such as arthrocentesis.

Tissue-like structures have been described in SF and SF sediments. Rice bodies are fibrin-impregnated, macroscopically visible structures that are believed to be derived from necrotic villi [3, 4]. Microscopic analysis sometimes reveals aggregates of cells entrapped in the fibrin strands typical of inflamed SF. Denser, hyalinized particles that occur preferentially in rheumatoid arthritis (RA) SF have been termed microscopic rice bodies [5]. In our own experience, looser groups of exudate cells, apparently held together by a mesh of fibrin (‘exudative cell clumps’), sometimes resemble microscopic rice bodies, but to our knowledge have not been studied systematically.

Intact pieces of synovium or synovial lining have been documented in SF [6] but have not been well characterized. Here we describe a protocol for the reliable detection of synovial lining fragments in inflammatory and non-inflammatory SF. This protocol employs crucial steps not used in traditional SF analysis, which explains why the fragments have largely gone unappreciated in the past. They may afford opportunities to study aspects of the synovial lining without biopsy and without introducing changes that result from propagating cells in culture. Moreover, they may contribute to persistent synovial inflammation or joint damage by releasing cytokines or apoptotic cell debris into the SF.

Materials and methods

Fluid collection and sample preparation

Thirty-nine consecutive joint and bursa effusions from adult patients seen in the rheumatology out-patient department of the Philadelphia VA Medical Center were aspirated as clinically indicated, using a standard sterile procedure [7] and a 20 gauge needle. The study was approved by the local ethics committee (Institutional Review Board). Informed consent was waived because: (i) specimens were used that would otherwise have been discarded; and (ii) no personally identifiable information was retained with the specimens. All specimens were collected into heparinized Vacutainers and processed within 4h. White blood cell (WBC) and differential counts were determined with a haemocytometer and Wright-stained cell smears, respectively. Wet drop
preparations were examined for tissue fragments and crystals with and without alizarin red S stain [7, 8]. Inflammatory fluids (WBC >2000/mm³) were centrifuged at 170 g for 10 min; the resulting pellets were resuspended in 2–5 ml of an ethanol-based fixation fluid (CytoRich Red Collection Fluid™; Thermo Shandon), left at room temperature for 15–30 min to allow fixation and erythrocyte lysis, and then centrifuged and washed twice in fixation fluid. The final pellets were resuspended in 2–5 ml fixation fluid. In addition, in the more viscous non-inflammatory fluids (WBC < 2000/mm³) mucin was lysed with dithiothreitol (Stat-Pack Sputolysin™; Caldon Biotech) for 30 min before fixation, followed by centrifugation at 600 g for 10 min. An aliquot of each specimen was centrifuged onto a coated cytospin slide (Cytoslide™; Thermo Electron Corporation) by rotating in a Shandon Cytoslide™ device at 800 r.p.m. for 10 min. The input volume (10–100 µl) was adjusted to result in a single layer of cells and tissue fragments on the slide. At least three cytospins were prepared from each specimen. The presence of a tissue fragment was defined as an aggregate of five or more mononuclear cells on one or more cytospins prepared from a given specimen. A diagram of the detection protocol is depicted in Fig. 1.

**Histochemistry and immunohistochemistry**

All slides were examined by standard and polarized light microscopy. Wright, alizarin red S and haematoxylin/eosin (H&E) stains were performed according to standard protocols [7, 8], and the pentachrome stain according to the modified Russell–Movat method [9]. Paraffin cell blocks were made from 11 specimens that contained easily visible, tight pellets after low-speed centrifugation. An automated immunostaining system (Ventana BenchMark IHC/ISH Staining Module) was used for immunostaining, using a commercial anti-CD68 (KP-1) primary antibody (Ventana Confirm™) and the Ventana iView DAB Detection Kit.

**Electron microscopy**

SF pellets were obtained by low-speed centrifugation, fixed briefly, disrupted into fragments of approximately 1×1 mm, fixed for 4 h in 0.5× Karnovsky’s paraformaldehyde–glutaraldehyde fixative, and then sectioned and processed as described previously [10]. A Zeiss EM-10 electron microscope with a 60-kV beam was used.

**Statistical analysis**

Linear regression was performed using the on-line program available at http://home.ubalt.edu/ntsbarsh/Business-stat/otherapplets/Regression.htm.

**Results**

**Clinical characteristics of patients and SFs**

Clinical diagnoses were: osteoarthritis (OA), eight cases; OA with calcium pyrophosphate dihydrate (CPPD) crystals, seven; RA, six; interval gout, five; gout with flare, four; olecranon bursitis, three; psoriatic arthritis (PsA), two; undifferentiated arthritis, two; pseudogout, one; and Lyme disease, one. Thirty-eight patients (97%) were male. Age ranged from 48 to 84 yr (mean, 66). Thirty-four effusions were obtained from knee joints, one from the acromioclavicular joint, and four from the olecranon bursa. Patient data and results of routine SF analysis are summarized in Table 1.

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**Table 1. Characteristics of patients and effusions**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>SF WBC count (/mm³)</th>
<th>Mean</th>
<th>Range</th>
<th>No. (%) with fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA</td>
<td>8</td>
<td>349</td>
<td>50–1000</td>
<td>5 (63%)</td>
<td></td>
</tr>
<tr>
<td>OA with CPPD</td>
<td>7</td>
<td>178</td>
<td>50–450</td>
<td>5 (83%)</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>6</td>
<td>13100</td>
<td>3400–40 000</td>
<td>2 (40%)</td>
<td></td>
</tr>
<tr>
<td>Gout, interval</td>
<td>5</td>
<td>240</td>
<td>50–800</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td>Gout, flare</td>
<td>4</td>
<td>9175</td>
<td>4500–18 700</td>
<td>2 (50%)</td>
<td></td>
</tr>
<tr>
<td>Bursitis</td>
<td>3</td>
<td>683</td>
<td>100–1850</td>
<td>2 (67%)</td>
<td></td>
</tr>
<tr>
<td>PsA</td>
<td>2</td>
<td>4525</td>
<td>3750–5300</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated arthritis</td>
<td>2</td>
<td>12075</td>
<td>8850–15 300</td>
<td>2 (100%)</td>
<td></td>
</tr>
<tr>
<td>Pseudogout</td>
<td>1</td>
<td>150</td>
<td>100–1500</td>
<td>1 (100%)</td>
<td></td>
</tr>
<tr>
<td>Lyme disease (treated)</td>
<td>1</td>
<td>150</td>
<td>1</td>
<td>1 (100%)</td>
<td></td>
</tr>
<tr>
<td>All non-inflammatory</td>
<td>24</td>
<td>310</td>
<td>50–1850</td>
<td>17 (71%)</td>
<td></td>
</tr>
<tr>
<td>All inflammatory</td>
<td>15</td>
<td>11233</td>
<td>3400–40 000</td>
<td>12 (80%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td></td>
<td>50–40 000</td>
<td>29 (74%)</td>
<td></td>
</tr>
</tbody>
</table>
Identification of SF tissue fragments in cytospins and SF sediments

Tissue fragments were not seen in Wright-stained cell smears and only rarely (<1%) in wet drop preparations. When SF was centrifuged onto coated cytospin slides, Wright stains could not be interpreted because of abundant, fixed fibrin deposits resulting from the ethanol fixation (data not shown). In contrast, the H&E stain revealed well-organized, variously sized, elongate and spindle-shaped tissue fragments, which consisted of large mononuclear cells with pale nuclei and abundant cytoplasm (Fig. 2A and B). These ranged in size from five or six to several hundred cells and were detected in bursitis and all types of arthropathies. The detection rate (percentage of specimens with the same clinical diagnosis in which at least one lining fragment was detected) ranged from 40 to 100% (Table 1), with an average of 71% for all non-inflammatory and 80% for all inflammatory fluids. Linear regression analysis revealed no significant correlation between the detection rate and the degree of inflammation, as measured by SF cell count number \( r = 0.31, P = 0.39 \). The total number of fragments per SF aspirate (extrapolated from the volume of fixation fluid in which the fragments were suspended, the input volume into the cytospins, and the number of fragments with more than five or six cells per cytospin) ranged between 15 and over 2500. The relative size distribution of the fragments was not determined. The fragments were also detected when centrifuged SF pellets were embedded in paraffin, the resulting blocks sectioned and stained with H&E (Fig. 2C). Their histological appearance and the almost complete absence of blood vessels and other subintimal structures suggested that these fragments were derived from the synovial lining (intima). These fragments were easily distinguished from exudative cell clumps, which appeared as loose, poorly organized aggregates containing heterogeneous inflammatory cells embedded in a loose matrix (Fig. 2E).

In sections from a synovial biopsy specimen from a different individual, synovial lining fragments which looked very similar to those in Fig. 2B and C were seen adjacent to or loosely attached to synovium (Fig. 2G and H).

Demonstration of intact synovial lining cells in the tissue fragment

To verify the identity of these fragments, pentachrome staining was performed. Diffuse red cytoplasmic staining was seen, and also small, yellow-stained collagen-containing areas (Fig. 2D, solid arrows). This appearance is characteristic of the synovial lining. Indeed, a tissue fragment found in a closed needle synovial biopsy specimen exhibited a similar staining pattern (Fig. 2H). The pentachrome stain also differentiated the tissue fragments from exudative cell clumps: the latter consisted of large, polymorphonuclear neutrophils and small mononuclear cells embedded in a poorly organized matrix of fibrin (red), mucin (blue) and, less, collagen (yellow) (Fig. 2F). Fragments from one patient were examined by EM, which revealed juxtaposed cells with the morphological features of type A (macrophage-like) and type B (fibroblast-like) synovial lining cells (Fig. 3), as expected in the synovial lining [11].

Expression of CD68 in the tissue fragments

In another attempt to verify the identity of these fragments, we wished to determine whether they express CD68, a marker commonly expressed by macrophage-like (type A) synoviocytes [12]. Immunoperoxidase staining revealed intense CD68 staining in a subset of the cells (Fig. 2I), as typically seen in the synovial lining.
Fig. 3. Transmission EM. (A) A cell with prominent vacuoles and dense chromatin probably corresponds to a macrophage-like, type A synoviocyte. Immediately adjacent, a cytoplasmic process with the typical features of a fibroblast-like synoviocyte (prominent rough endoplasmic reticulum and mitochondria) is seen. M, macrophage-like synoviocyte; v, vacuole; F, fibroblast-like synoviocyte. Original magnification, 12,500×. (B) Further magnification, illustrating rough endoplasmic reticulum (rER) and mitochondria-rich cell process characteristic of fibroblast-like synoviocytes. mt, mitochondria. Original magnification, 47,500×.

Discussion

Detection and characterization of synovial lining fragments

We have detected small tissue fragments in SF. Even though these fragments have gone essentially unnoticed in the past, we have detected them surprisingly frequently and easily. We attribute this ease of detection to four crucial steps in our protocol, which represent major differences from traditional SF analysis: (i) ethanol fixation; (ii) mucolysis of viscous specimens; (iii) use of cytospins; and (iv) staining with H&E instead of the commonly used Wright stain. We identified the fragments as synovial lining tissue by their characteristic light microscopic appearance, the detection of fibroblast-like and macrophage-like synoviocytes by EM, and by the detection of CD68 in a pattern typical of hyperplastic synovial lining. The results of the pentachrome stain, too, support the origin of the fragments from the synovial lining: small, scattered collagen-containing areas were detected, as may be seen in the synovial lining (Fig. 2D). We also clearly differentiated these fragments from rice bodies and exudative cell clumps: exudative cell clumps have a much looser, less differentiated appearance than rice bodies, and thus, we could distinguish these fragments from rice bodies and exudative cell clumps.

Origin of the synovial tissue fragments

In our experience, tissue fragments are also seen in some unstained wet preparations from traumatic arthrocenteses, which required multiple needle passages. Thus, traumatic dislodgement may be a cause in some cases. However, traumatic arthrocentesis occurs infrequently, and most of the specimens in this study were obtained easily during first needle passage and were not bloody. The detection of these tissue fragments barely attached to intact synovium (e.g. Fig. 2G and H) suggests a different mechanism of origin for the majority of them. It is conceivable that mechanical shearing forces or natural remodelling could dislodge structures like this one into the SF. Most of the fragments seen are significantly hyperplastic. Thus, avulsion may also be the result of relative tissue weakening from lack of nutrients.

Potential uses of the synovial tissue fragments

Paraffin-embedded SF sediments containing the fragments were easily used for immunohistochemistry (Fig. 2I), demonstrating that some, if not all, proteins and antigenic epitopes are preserved. Moreover, the fragments were detected in all arthropathies studied. We had arbitrarily determined to analyse three cytospins per specimen, which led to detection in all arthropathies studied, with a detection rate of at least 40% in the specimens of the same diagnosis. Conceivably, analysing higher numbers of cytospins per specimen could increase the detection rate further. Taken together, these observations suggest that the fragments could potentially be used to study protein expression changes in the synovial lining in arthropathies and bursitis. However, further studies are first needed to (i) determine if the amount of tissue obtained with these fragments suffices reliably for such analysis, and (ii) to determine how closely the fragments resemble the resident synovial lining at the molecular level. Since it does not require propagation of the tissue by culture, one approach to the latter question would be to isolate the tissue fragment and the resident synovial lining by laser capture microdissection, and then compare their PCR- or microarray-based transcript profiles with each other.

Potential significance of the synovial tissue fragments in persistent synovitis

SF fibroblasts display highly activated phenotypes and mediate cartilage destruction, and it has been suggested that they contribute significantly to inflammation in the synovium [1, 2]. Owing to their size, the tissue fragments would be expected to be relatively resistant to phagocytosis and may thus persist in SF for some time. We speculate that they may continue to secrete proinflammatory substances and release apoptotic cell debris, thus perpetuating an inflammatory or destructive process. Indeed, microparticles from apoptotic cells were recently shown to induce the synthesis of matrix metalloproteinases and proinflammatory cytokines in synovial fibroblasts [13]. This also agrees well with the observation that lavage at the time of intra-articular corticosteroid injection enhances the efficacy of the injection, presumably by removing proinflammatory substances and debris from the joint cavity [14, 15]. As seen in Table 1, we detected these fragments in patients with joint effusions but non-inflammatory SF cell counts. In these cases, the tissue fragments may have contributed to the persistent joint effusion.

We realize the limitations of this study. The fixation fluid lysed red blood cells, and we may thus have failed to notice some small blood vessels in the fragments. Immunostaining for vascular endothelial cells, for instance with anti-von Willebrand factor or anti-CD31 antibodies, might have led to the detection of some small blood vessels and revealed the presence of more subintimal tissue than suggested by our analysis. We have not attempted to demonstrate the presence of synovial lining fibroblasts by immunohistochemistry. This could be done using a specific marker for these cells, such as uridine diphosphoglucose dehydrogenase or complement decay accelerating factor [16]. However, the presence of large numbers of CD68-negative cells, their characteristic light microscopic appearance, and the detection of cells with the typical EM features of synovial lining fibroblasts (Fig. 3) all strongly suggest that synovial lining fibroblasts are contained in the tissue fragments.
Acknowledgements

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References