Subintimal Ki-67 as a synovial tissue biomarker for inflammatory arthropathies

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Abstract

OBJECTIVES: Ki-67 is expressed in the nuclei of dividing cells and can be used to assess proliferation of synovial inflammatory and stromal cells. We evaluated subintimal Ki-67+ cell density as a tissue biomarker for inflammatory arthropathies and compared it to subintimal CD68, a synovial biomarker of RA. METHODS: Subintimal Ki-67+ and CD68+ cell densities were measured immunohistochemically in synovial specimens obtained from patients with rheumatoid arthritis (RA; n = 19), osteoarthritis (OA; n = 18), "non-inflammatory" orthopaedic arthropathies (avascular necrosis, meniscus injury, femur fracture; n = 16), chronic septic arthritis (n = 9), and histologically normal synovium (n = 10). RESULTS: were correlated with a histological synovitis score. Utilising the areas under receiver operating characteristic curves (AUCs), we compared the abilities of Ki-67 and CD68 to differentiate among these arthropathies. Results: Ki-67 was expressed widely in the subintimal of inflamed specimens and in RA pannus invading hard tissues. Compared to normal controls, it was highly overexpressed in RA (26.6-fold) and chronic septic arthritis (55-fold), and mildly elevated in OA (3.9-fold) and orthopaedic arthropathies (2.1-fold). Ki-67 and CD68 differentiated similarly well between RA and OA (AUC: Ki-67 = 0.91, CD68 = 0.94), Ki-67 better between chronic septic arthritis and RA, and CD68 better between OA and normal controls. Ki-67 (r = 0.80) and CD68 (r = 0.79) correlated positively with the synovitis score. CONCLUSIONS: Subintimal Ki-67 was overexpressed in inflammatory arthropathies, distinguished among differentially inflamed arthropathies, and correlated positively with the histological severity of synovitis. It may prove useful in synovial tissue classification and as a synovial marker of disease activity in clinical trials when biopsies are available.
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ABSTRACT

Objectives: Ki-67 is expressed in the nuclei of dividing cells and can be used to assess proliferation of synovial inflammatory and stromal cells. We evaluated subintimal Ki-67+ cell density as a tissue biomarker for inflammatory arthropathies and compared it to subintimal CD68, a synovial biomarker of RA.

Methods: Subintimal Ki-67+ and CD68+ cell densities were measured immunohistochemically in synovial specimens obtained from patients with rheumatoid arthritis (RA; n = 19), osteoarthritis (OA; n = 18), “non-inflammatory” orthopaedic arthropathies (vasculitis necrosis, meniscus injury, femur fracture; n = 16), chronic septic arthritis (n = 9), and histologically normal synovium (n = 10). Results were correlated with a histological synovitis score. Utilising the areas under receiver operating characteristic curves (AUCs), we compared the abilities of Ki-67 and CD68 to differentiate among these arthropathies.

Results: Ki-67 was expressed widely in the subintima of inflamed specimens and in RA pannus invading hard tissues. Compared to normal controls, it was highly overexpressed in RA (26.6-fold) and chronic septic arthritis (55-fold), and mildly elevated in OA (3.9-fold) and orthopaedic arthropathies (2.1-fold). Ki-67 and CD68 differentiated similarly well between RA and OA (AUC: Ki-67 = 0.91, CD68 = 0.94), Ki-67 better between chronic septic arthritis (n = 9), and histologically normal synovium (n = 10). Results were correlated with a histological synovitis score. Utilising the areas under receiver operating characteristic curves (AUCs), we compared the abilities of Ki-67 and CD68 to differentiate among these arthropathies.

Conclusions: Subintimal Ki-67 was overexpressed in inflammatory arthropathies, distinguished among differentially inflamed arthropathies, and correlated positively with the histological severity of synovitis. It may prove useful in synovial tissue classification and as a synovial marker of disease activity in clinical trials when biopsies are available.

The nuclear antigen Ki-67 is closely associated with cellular proliferation in that it is specifically expressed in G1, S, G2, and M phases of the cell cycle, but not in quiescent (G0) cells. Ultrastructural studies have shown that it is a critical component of the formation of the perichromosomal scaffold and supports the nucleoli during cell proliferation. Due to this close association with cell division, Ki-67 is widely used in tumour classification, but it has also been used to characterise proliferating cells in diseased synovium, namely in the hyperplastic synovial lining and in subintimal lymphocyte populations.

A principal difference between Ki-67 and other currently used histological synovial biomarkers (eg, CD68 or CD38) lies in the fact that it may be expressed by any dividing synovial cell and therefore does not measure the numbers of a given cell type but, rather, the activity of a tissue-wide process. Its expression would therefore reflect proliferation of resident stromal cells as well as of infiltrating inflammatory cells. The exclusive nuclear localisation of Ki-67 represents an added benefit for histomorphometric analyses in that it facilitates differentiating positive staining cells from negative staining ones, even in densely packed cell clusters. Recently, the search for synovial biomarkers of disease activity and treatment response in inflammatory arthropathies has focused on infiltrating inflammatory cells in the synovial subintima. However, the potential of subintimal Ki-67 as a tissue biomarker in terms of disease activity and tissue diagnosis has not been studied. In the present study, we have therefore evaluated the potential of subintimal Ki-67 to distinguish among various arthropathies and assessed its correlation with disease activity as measured by histological abnormalities of synovitis.

MATERIALS AND METHODS

Synovial tissue specimens were obtained by closed needle biopsy or surgically at the time of arthroplasty. The following specimen groups were studied: rheumatoid arthritis (RA) with active disease despite disease modifying anti-rheumatic drug (DMARD) treatment (n = 19), chronic septic arthritis proven by positive bacterial culture (n = 9; disease duration in these specimens was estimated from retrospective chart review and was between ≥4 weeks and 1 year), osteoarthritis (OA; n = 18), and 16 specimens from orthopaedically-treated arthropathies (OraH, consisting of femur fracture (n = 3), meniscus injury (n = 10), and avascular necrosis of the femoral head (n = 3)) as “less inflamed” disease controls. These orthopaedic arthropathies form a heterogeneous group characterised by mild synovitis, similar to that often seen in OA. Ten histologically unremarkable specimens from patients with non-inflammatory knee pain were included as “normal” controls.

The absence of appreciable numbers of inflammatory cells was verified in these “normal” specimens by immunostaining for CD3, CD20, CD58 and CD68. Expression at sites of pannus invading cartilage and bone was determined in one RA specimen obtained at the time of arthroplasty. Tissues were fixed in formalin and embedded in paraffin according to standard practice. Sections (5-μm thick) were prepared on a microtome and immunostained on an automated staining system (Ventana Benchmark, Ventana, Tucson Arizona, USA) for Ki-67 (clone K-2) or CD68 (KP-1), using pre-diluted commercial antibody preparations (Ventana). General antigen preservation was tested...
in all specimens by staining for von Willebrand Factor (vWF). One specimen was excluded from the analysis because of lack of vWF staining even though blood vessels were present, suggesting poor antigen preservation. The numbers of Ki-67 or CD68 expressing cells per high power field (hpf, 400 ×) were determined in 5-12 fields per specimen by manual cell counting as described. Only fields with clearly recognisable lining and subintimal vasculature were included. All results were converted to densities per mm² using the formula positive staining cells/mm² = (positive staining cells/400 × field) × 6.29.

Using the values for subintimal Ki-67+ or CD68+ cell densities, the sensitivities and specificities of each marker in differentiating between any two arthropathies were determined for all available values. These were then plotted as receiver operating characteristic curves (ROCs), where the area under the curve (AUC) is a numeric measure of the ability of a diagnostic test to distinguish between two diagnostic possibilities. The AUCs and corresponding p values were calculated with the SPSS biostatistical software (SPSS Inc., Chicago, Illinois, USA). Using the grading system (“synovitis score”) described by Krenn et al., the histopathological severity of synovitis was determined in 37 specimens (RA, n = 14; OA, n = 10; OrthA, n = 5; and “normal”, n = 8) in which Ki-67 and CD68 expression had been determined immunohistochemically. These specimens were selected to encompass a broad range of inflammation and to contain members of all specimen groups, except chronic septic arthritis, for which this score has not been validated. In order to account for the relatively small sizes of some of the needle biopsies, the grading system was modified insofar as the score for each specimen was derived from the

Figure 1 Representative immunohistochemical findings. Diaminobenzidine (brown; nearly black in highly expressing nuclei) was used as chromogen. SeA, chronic septic arthritis. A and B. Diffusely infiltrating abundant Ki-67+ cells in chronic septic arthritis. Ki-67+ lymphoid follicles were also seen in one of the specimens (not shown here). C. RA. Classic lymphoid follicle with a Ki-67+ germinal centre surrounded by a transitional zone containing further Ki-67+ lymphocytes. Superficial subintima; the lining is barely visible at the right margin of the image. D. Ki-67+ cells in a plasma cell-rich infiltrate in an RA villus. A few Ki-67+ cells are seen in the adjacent hyperplastic lining (arrows). li, lining. E. OA. A solitary Ki-67+ cell at the junction between subintima and lining. F. OA. A small number of Ki-67+ cells in the subintima. G. Normal synovium. Ki-67+ adipocytes (some marked by arrows). H. Normal synovium. An uncommon example of Ki-67 expression in non-adipocyte stromal cells (arrows).
average of 3–10 microscopic fields (200×). Subintimal Ki-67 or CD68 cell densities were correlated separately with the synovitis score by linear regression. Statistical significance was determined by analysis of variance (ANOVA), and sample size calculation with the open access software program PS vs 2.1.31 (available at http://biostat.mc.vanderbilt.edu).

RESULTS

Immunohistochemical detection of Ki-67 in synovium and at the pannus/hard tissue interface

Ki-67 was detected in all specimens with adequate antigen preservation, as determined by vascular staining for vWF. Consistent with previous reports,2-5 Ki-67 was expressed in lining and subintima (fig 1). Ki-67+ cells were essentially absent from the lining of normal controls (fig 1G,H) and were detected rarely in the OA lining. Even though they were more common, Ki-67 expressing cells were relatively sparse even in the hyperplastic lining of RA (eg, fig 1D). In the subintima, Ki-67 appeared to be much more common in the inflamed specimens, particularly in those obtained from patients with chronic septic arthritis or RA (fig 1A–D). Highest concentrations were seen in and near lymphoid aggregates, which were most common in RA. Figure 1C illustrates a lymphoid follicle seen in an RA specimen, with a cluster of Ki-67+ cells defining the germinal centre. Figure 1D shows a different RA specimen, which contained scattered Ki-67+ cells in a plasma cell-rich subintimal infiltrate as well as positively staining foci in the hyperplastic lining. Only solitary Ki-67+ cells typically occurred in OA (fig 1E–F). Ki-67 was also detected in adipose tissue of minimally inflamed and normal specimens, and adipocytes often represented the only Ki-67+ cells in the normal controls (fig 1G). These were excluded from the semi-quantitative analysis. Whereas it did not seem to occur in chondrocytes, Ki-67 was detected in cells at the leading front of RA pannus invading surface cartilage (fig 2A) and, more frequently, in vascularised pannus that had penetrated into subchondral bone (fig 2B).

Semi-quantitative differences among the specimen groups in subintimal Ki-67+ and CD68+ cell densities

We then determined the relative expression of subintimal Ki-67 and CD68 in the arthropathies included in this study (fig 3). The x-fold expression differences and p values are summarised in table 1. The mean Ki-67+ cell density was lower than the mean CD68+ cell density in all specimen groups. The smallest difference was seen in chronic septic arthritis, which contained only 33% fewer Ki-67+ than CD68+ cells, and the largest in OA, OrthA and normal controls (80–84% fewer Ki-67+ cells). The lowest mean Ki-67+ cell density occurred in the normal specimens (fig 3). Mildly elevated mean densities were found in OrthA and OA, and even higher values in RA. Notably, the highest mean density was found in chronic septic arthritis, resulting in a mean 55-fold overexpression compared to the normal controls. CD68+ cell densities followed a similar trend, except that there was no significant difference between RA and SeA and that the differences between RA and SeA vs OA or normals were smaller than those measured with Ki-67 (table 1).

Given the relatively small sample sizes in this study, a sample size calculation was performed to determine minimal sample sizes needed to differentiate RA from the other arthropathies. With the exception of CD68 in the comparison RA vs SeA (whose x-fold expression difference was marginal and not statistically significant), the predicted sample sizes fell within the sizes of the specimen groups used (table 2).

Subintimal Ki-67+ cell density in the differential histological classification of synovitis

Based on the expression data summarised in fig 3, we then computed sensitivities and specificities for all available Ki-67+ or CD68+ cell densities in the differentiation between any two arthropathies. The areas under the corresponding ROC curves were used to compare the performance of Ki-67 and CD68 as diagnostic markers (table 1, columns 5 and 6). Consistent with the dramatic overexpression of both markers in RA and chronic septic arthritis (fig 3 and table 1), they distinguished between RA or chronic septic arthritis and the normal controls with the maximally possible AUCs of 1. Both factors also had similar AUCs for distinguishing between OA and RA and between OA and chronic septic arthritis. However, they differed greatly in the ability to differentiate between other specimen groups: CD68 distinguished better between OA or OrthA and normal controls, whereas Ki-67 differentiated better between chronic septic arthritis and RA. Of note, the differences between Ki-67 and CD68 in AUCs did not always correspond to their x-fold differences in positive staining cell densities. For example, in the comparison chronic septic arthritis/OA, the x-fold increase of Ki-67+ cell density in chronic septic arthritis (with respect to OA) was 3.9-fold higher than that of CD68 (14.1 vs 3.6), whereas their AUCs were nearly identical. Likewise, in the comparison RA/OA, the x-fold increase in Ki-67+ and CD68+ cell densities was 1.8-fold higher than the increase in CD68 (6.8 vs 3.8), but its AUC was slightly lower (table 1).

Figure 2 Expression of Ki-67 in RA pannus invading hard tissues. A. A small number of Ki-67+ cells in pannus invading surface cartilage. P, pannus; C, cartilage. B. Higher density of Ki-67+ cells in vascularised pannus invading subchondral bone. The arrows point to Ki-67+ cells located at the pannus/bone interface. B, bone.
Ki-67 and CD68: coincidence with synovitis

Ki-67+ and CD68+ cell densities were compared to synovitis scores in 37 specimens. Ki-67+ subintimal cell densities correlated strongly with the synovitis score (fig 4). Its correlation coefficient (r) of 0.80 was essentially identical to that of CD68. The present study also demonstrates that, compared to OrthA and OA, this agrees with the notion that at least mild inflammation occurs often in OA synovium. We have recently shown that “non-inflammatory” orthopaedic arthropathies form a heterogeneous group of mildly inflamed specimens, similar to OA.15 This finding is supported by the current results that the numbers of Ki-67+ cells were similar in OrthA and OA, but higher in both than in the “normal” controls (fig 5). Surprisingly, the highest mean Ki-67+ cell density was detected in chronic septic arthritis. This is likely due to the presence of

**Table 1** Ki-67 and CD68+ cell densities and AUCs* as diagnostic biomarkers in synovium

<table>
<thead>
<tr>
<th>Comparison</th>
<th>x-Fold differences in positive staining cells/mm²</th>
<th>AUCKi-67 (p value)</th>
<th>AUCCD68 (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA vs normal</td>
<td>3.9 (0.028)</td>
<td>3.7 (0.003)</td>
<td>0.78 (0.014)</td>
</tr>
<tr>
<td>OrthA vs normal</td>
<td>2.1 (0.14)</td>
<td>2.5 (0.06)</td>
<td>0.61 (0.34)</td>
</tr>
<tr>
<td>RA vs normal</td>
<td>2.66 (0.001)</td>
<td>13.9 (&lt;0.001)</td>
<td>1 (&lt;0.001)</td>
</tr>
<tr>
<td>SeA vs normal</td>
<td>55.0 (0.001)</td>
<td>13.4 (&lt;0.001)</td>
<td>1 (&lt;0.001)</td>
</tr>
<tr>
<td>RA vs OA</td>
<td>6.8 (&lt;0.001)</td>
<td>3.8 (&lt;0.001)</td>
<td>0.91 (&lt;0.001)</td>
</tr>
<tr>
<td>SeA vs OA</td>
<td>14.1 (&lt;0.001)</td>
<td>3.6 (&lt;0.001)</td>
<td>1 (&lt;0.001)</td>
</tr>
<tr>
<td>SeA vs RA</td>
<td>2.1 (0.035)</td>
<td>0.96 (0.82)</td>
<td>0.79 (0.015)</td>
</tr>
</tbody>
</table>

* AUC, area under the curve; derived from the corresponding receiver operating characteristic (ROC) curves.

**Table 2** Sample size calculation comparing Ki-67 and CD68*

<table>
<thead>
<tr>
<th></th>
<th>RA vs SeA</th>
<th>RA vs OA</th>
<th>RA vs OrthA</th>
<th>RA vs normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67</td>
<td>9</td>
<td>7</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>CD68</td>
<td>1612</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

* Minimal sample sizes needed to achieve \( \alpha < 0.05 \) and power \( \beta > 0.80 \).

**DISCUSSION**

In this study, we have quantified further the association of subintimal Ki-67 expression with synovitis, have begun to evaluate its potential as a tissue biomarker and, notably, found a strong correlation with the histological degree of synovitis. Subintimal Ki-67+ cell densities have not been quantified systematically in cohorts of differentially inflamed arthropathies. The fundamental study by Nykänen et al16 identified B and T lymphocytes as major proliferating cell types in RA synovium, a finding that was later confirmed by double immunohistochemistry with lymphocyte surface markers and Ki-67.2 To our knowledge, only one study, which focused on Ki-67 expression in the synovial lining, attempted to identify systematic differences among different arthropathies.4 These authors showed that Ki-67 positive cells tended to be more frequent in the lining of RA synovium than in reactive arthritis or psoriatic arthritis, although these differences were not statistically significant. Consistent with this trend toward higher Ki-67 expression in RA, our present analysis of Ki-67 expression in the subintima revealed a remarkable, statistically significant overexpression in RA, compared to the less inflamed disease controls and normal specimens (figs 1 and 5; table 1).

The present study also demonstrates that, compared to histologically normal synovium, subintimal Ki-67+ cell density is increased in the less inflamed synovial specimens such as OA and OrthA. This agrees with the notion that mild inflammation is often present in OA synovium. We have recently shown that “non-inflammatory” orthopaedic arthropathies form a heterogeneous group of mildly inflamed specimens, similar to OA.15 This finding is supported by the current results that the numbers of Ki-67+ cells were similar in OrthA and OA, but higher in both than in the “normal” controls (fig 5). Surprisingly, the highest mean Ki-67+ cell density was detected in chronic septic arthritis. This is likely due to the presence of
The high numbers of Ki-67+ cells in the RA specimens agree well with previous reports of increased cell division in RA synovium and at the pannus–hard tissues interface. Subintimal Ki-67+ expression also correlated strongly with histological alterations associated with synovitis, indicating that it is pathogenetically relevant. For histopathologic grading, we chose the scoring system by Krenn et al because of its broad dynamic range, which allows for the detection of inflammation-associated changes in high-grade synovitis, but also in the mildly inflamed OA and OrthA specimens. The validity of this scoring system in the present study was confirmed by the fact that the mean synovitis scores of our RA and OA specimen groups were very similar to those reported by Krenn et al. Ki-67+ cells correlated nearly as strongly with the synovitis score as CD68+ subintimal cells, a marker strongly associated with a diagnosis of RA and currently the best validated synovial marker for a positive response to pharmacologic treatment of RA.

Thus, Ki-67 promises to be useful as a marker for similar clinical aspects of inflammatory arthropathies. It will now be important to perform additional studies, which evaluate the expression of Ki-67 in other inflammatory arthropathies such as psoriatic and reactive arthritis, and its correlation with a response to treatment interventions and other clinical outcomes. The search for synovial biomarkers in OA has been dissatisfying. Considering the correlation of Ki-67 with the synovitis score in the lower part of the spectrum (fig 4) and its small but significant overexpression in OA compared to normal controls (fig 3), it also merits further investigation as a synovial biomarker in less inflamed arthropathies such as OA.

Considering (1) the ease with which Ki-67+ cells can be distinguished from Ki-67- negative cells, and (2) the large expression differences obtained by our simple scoring method, subintimal Ki-67+ cell density should prove useful as a powerful but easy-to-use synovial marker for a variety of questions. By extension, other constitutively nuclear proteins, such as many transcription factors, will likely possess similar practical advantages and may also turn out to be useful synovial biomarkers.
REFERENCES


