Measurement of cytokine and adhesion molecule expression in synovial tissue by digital image analysis

M C Kraan, M D Smith, H Weedon, M J Ahern, F C Breedveld, P P Tak

Abstract

Objective—Digital image analysis (DIA) offers the opportunity to quantify the stained area and staining intensity when synovial tissue (ST) is investigated by immunohistochemical analysis. This study aimed at determining the sensitivity of DIA compared with semiquantitative analysis (SQA).

Methods—Paired ST samples were obtained from the knee joint of 10 patients with rheumatoid arthritis (RA) with active disease and after follow up when complete clinical remission was achieved. ST samples of 10 subjects with non-inflammatory knee pain served as controls. Immunohistochemistry with antibodies against interleukin 1β (IL1β) and vascular cell adhesion molecule 1 (VCAM-1) was applied using two staining protocols with 3-amino-9-ethylcarbazole (AEC) or 3,3′-diaminobenzidine (DAB) as dye. All sections were analysed semiquantitatively (0–4) and DIA of up to a maximum of 60 high power fields (HPF). The average integrated optical density was calculated as the product of the stained area (corrected for total tissue area) and the optical density.

Results—Both SQA and DIA enabled the assessment of differences in IL1β and VCAM-1 expression between ST from active RA, RA in remission, and controls. SQA and DIA showed excellent correlations (IL1β $r_s=0.867; p<0.0001$; VCAM-1 $r_s=0.828; p<0.0001$). A limited analysis of one region with six HPF still allowed adequate discrimination compared with an extended analysis of three regions with a total of 60 HPF. In general, the red dye (AEC) resulted in better discrimination than the brown (DAB) staining.

Conclusion—DIA offers a reliable, reproducible, and sensitive analysis of ST sections stained for cytokines and adhesion molecules.

In contrast with standard light microscopy, immunohistochemistry has allowed the evaluation of the expression of adhesion molecules and cytokines in synovial tissue (ST). This offered new perspectives in studies on the aetiopathogenesis and treatment of rheumatoid arthritis (RA).

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### Statistical Analysis

Wilcoxon signed rank tests were used for the comparison of active disease and remission. Mann-Whitney tests were used to compare remission and controls. Spearman rank tests were used to calculate the correlations between the different acquisition and analysis techniques. The plot of the average and the difference of two digital image analysis procedures and the calculation of the correlation were performed as suggested by Bland and Altman.

### Results

#### Patient Demographics

The mean age of the 10 patients with RA (six male, four female) was 72 years (range 59–77). Five patients had erosive disease and five patients non-erosive disease; average disease duration was 54 months (range 1–216). The mean age of the control subjects (six male, four female) was 39 years (range 28–54). None of the 10 patients with RA had taken disease modifying antirheumatic drugs at the time of the first biopsy procedure.

#### Results of SQA and DIA

As shown in table 1, SQA of the sections stained for IL1β showed a significant difference between active disease and remission (p<0.005), whereas the scores for IL1β were similar for patients with RA in remission and controls. DIA showed a significant difference between active disease and remission for 60 HPF (p=0.005), 20 HPF (p=0.005), and six HPF (p<0.01). Comparison of the results of DIA between patients with RA in remission and controls yielded no significant differences.

**Table 1**  Mean score (standard error of the mean) of semiquantitative analysis (SQA) and digital image analysis (DIA) on six high power fields (HPF) and 60 HPF for interleukin(IL)1β and vascular adhesion molecule (VCAM)-1. Depicted are the paired results for patients with active rheumatoid arthritis and after the achievement of clinical remission and healthy controls.

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**Figure 1** Plots of (A) semiquantitative analysis with digital image analysis of 20 high power fields (HPF) in one single region; (B) digital image analysis of 20 high power fields (HPF) in three regions with 20 high power fields (HPF) each (total 60 HPF); and (C) the difference between the two digital image analyses with the average between the two techniques as suggested by Bland and Altman.  

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SQA of the sections stained for VCAM-1 showed no significant difference between patients with active disease and patients in remission; in controls the scores were signifi-
cantly lower (p<0.05). DIA showed significant differences between active disease and remission for 60 HPF (p<0.05), 20 HPF (p=0.01), and six HPF (p<0.05). Comparison of the results of DIA between remission and controls also showed significant differences for 60 HPF (p=0.01), 20 HPF (p=0.007), and six HPF (p=0.005).

CORRELATIONS BETWEEN SQA AND DIFFERENT DIA METHODS
Figures 1A and B show that for IL1β, SQA was highly significantly correlated with DIA of 20 HPF (r=0.942, p<0.0001), and 20 HPF with 60 HPF (r=0.967, p<0.0001), respectively. A strong correlation was found between 60 HPF and 6 HPF (r=0.844, p<0.0001) as well. To investigate the effect of the limitation of the number of HPF in DIA we calculated the correlation between methods of measurement.12

The calculated correlation between 60 HPF and 20 HPF was −0.08, suggesting equal variance. Correlation between 60 HPF and 6 HPF was −0.834, suggesting a marked difference in the variability between subjects for the two methods despite the strong correlation. Figure 1C shows the plot of the difference against the average of both 60 HPF and 20 HPF. Similar results were found for VCAM-1.

Discussion
In this study we show that DIA and SQA give similar results for the analysis of both IL1β and VCAM expression in ST sections. Overall, DIA proved to be slightly more sensitive, especially in comparing remission with controls. Moreover, even a limited analysis of 20 HPF (or 2.5 mm² of ST) produced representative results.

To our knowledge this is the first paper comparing SQA with DIA for the analysis of cytokine and adhesion molecule expression in the synovial tissue of patients with RA. We chose to investigate the expression of IL1β in synovial tissue because this cytokine plays a part in both inflammation and destruction. Furthermore, it has been documented that joint destruction associated with RA can be found in patients in clinical remission and we expected this cytokine to be of special interest in our study group.13 DIA identified differences between controls and patients with RA in remission, but these changes were not statistically significant.

We investigated VCAM-1 based upon the role of the VCAM-1/VLA-4 ligand pair in the preferential recruitment of activated lymphocytes and monocytes from the circulation into the RA ST.14 Of interest, we noted an increased expression of VCAM-1 in patients with RA in remission. This observation is consistent with previous observations showing signs of activation in clinically unaffected joints.15

We conclude that the ability of DIA to quantify the expression of a marker offers a sensitive tool to determine protein expression in synovial tissue. Because it can be expected that DIA will be increasingly used for the evaluation of synovial tissue, further standardisation of the methodology is mandatory.