CONCISE REPORT

Low levels of apoptosis and high FLIP expression in early rheumatoid arthritis synovium

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Objectives: To define synovial apoptosis with respect to disease duration, inflammatory cell type, FLIP (FLICE-like inhibitory protein), and cytokines expression in patients with rheumatoid arthritis (RA).

Methods: Synovial biopsy specimens from 11 patients with longstanding RA (median disease duration 21 years) and eight with early RA (median disease duration five months) were investigated. Apoptosis (TUNEL method combined with morphological analysis), cell surface markers (CD3, CD68), cytokines (interleukin (IL) 1α, IL1β, tumour necrosis factor α, and IL6), and FLIP expression were evaluated. Computer assisted image analysis was used for quantification.

Results: The apoptosis level in RA synovium was significantly higher in the group of patients with longstanding RA than in the patients with early RA (8.8% *v* 0.6%, p=0.001), while the number of macrophages and FLIP expression were higher in the group with early disease than in the group with longstanding RA (16.2% *v* 8.3%, p=0.02 and 31.1% *v* 0.2%, p=0.001 respectively). All three markers correlated significantly with disease duration (R=–0.7, p<0.001 for FLIP, R=0.6, p=0.001 for apoptosis, and R=–0.5, p<0.05 for CD68). Cytokine expression and T cell score were not significantly different in early RA from longstanding RA. No differences were seen between patients treated or not treated with corticosteroids or between patients treated or not treated with disease modifying antirheumatic drugs.

Conclusions: The findings suggest that RA synovial macrophages are resistant to apoptosis in early RA and express high levels of FLIP. During natural or drug modified disease progression the apoptotic mechanism may be restored with a specific increase of synovial apoptosis in patients with longstanding arthritis.

Rheumatoid arthritis (RA) is an inflammatory disease characterised by synovial proliferation and excessive mononuclear infiltration partially due to an impaired apoptosis mechanism (for review see Nishioka *et al*[1]). Apoptosis is present in the rheumatoid synovial tissues with large variations between patients.[2] Adjuvant arthritis in rats, an experimental model of RA, is characterised by an increase in synovial apoptosis in late, chronic stages of the disease.[3] Several molecules have been proposed as potential modulators of the synovial apoptosis. In vitro, cytokines such as interleukin 1β (IL1β) and tumour necrosis factor α (TNFα) inhibit apoptosis of synovial cells.[4] Cellular and viral FLIP (FLICE-like inhibitory protein) inhibit death receptor mediated apoptosis and promote tumour progression.[5 6] Cellular FLIP is present in RA synovial biopsy specimens, mainly in macrophages.[7] In this study, to characterise synovial apoptosis modulation in RA further, we investigated the association between apoptosis and disease duration and features of the inflamed synovium—that is, inflammatory cell types, cytokines, and FLIP expression.

PATIENTS AND METHODS

Patients and samples

Nineteen patients, eight with early RA (four women and four men, median age 55, median disease duration five months) and 11 with longstanding RA (eight women and three men, median age 62, median disease duration 21 years), meeting the American College of Rheumatology criteria were recruited for this study. In the group with early RA none of the patients received corticosteroids, and two patients were treated with disease modifying antirheumatic drugs (DMARDs), auranofin and sulfasalazine, respectively. In the group with late RA five patients received corticosteroids to a maximum of 10 mg prednisolone daily and six were treated with DMARDs, receiving methotrexate (two patients), cyclosporin (two patients), hydroxychloroquine (one patient), or sulfasalazine (one patient).

Synovial biopsy specimens were obtained from all patients by arthroscopy in the group with early RA and by surgical resection in the group with longstanding RA. Serial cryostat sections (7 μm) were fixed for 20 minutes with 2% (vol/vol) formaldehyde or for 10 minutes with 100% acetone and stored at −70°C.

Immunohistochemistry

Synovial apoptosis was detected with a fluorescein labelled in situ cell death detection kit (Boehringer-Mannheim) combined with morphological evaluation, as previously described.[8] FLIP staining was performed using a monoclonal rat IgG2a antibody (804-129-C100 from Alexis Biochemicals, San Diego, USA) at a concentration of 2.5 μg/ml. Biopsy specimens were also evaluated for cytokine expression (IL1α, IL1β, IL6, TNFα) and cell surface markers (CD68, CD3), as previously described.[9] Negative controls using isotype matched IgG were included for each marker. Stained synovial biopsy sections were evaluated by computer assisted image analysis.[10] The area of positive staining was expressed as a percentage of the total cell area. Analysis of an entire tissue section typically involved 50–150 microscopic fields at a magnification of ×250.

Statistical analysis

Differences between groups were analysed by the Mann-Whitney U test and correlations between different variables were assessed by the Spearman rank correlation test. A p value <0.05 was considered significant.

Abbreviations: DMARDs, disease modifying antirheumatic drugs; FLIP, FLICE-like inhibitory protein; IL, interleukin; RA, rheumatoid arthritis; TNF, tumour necrosis factor

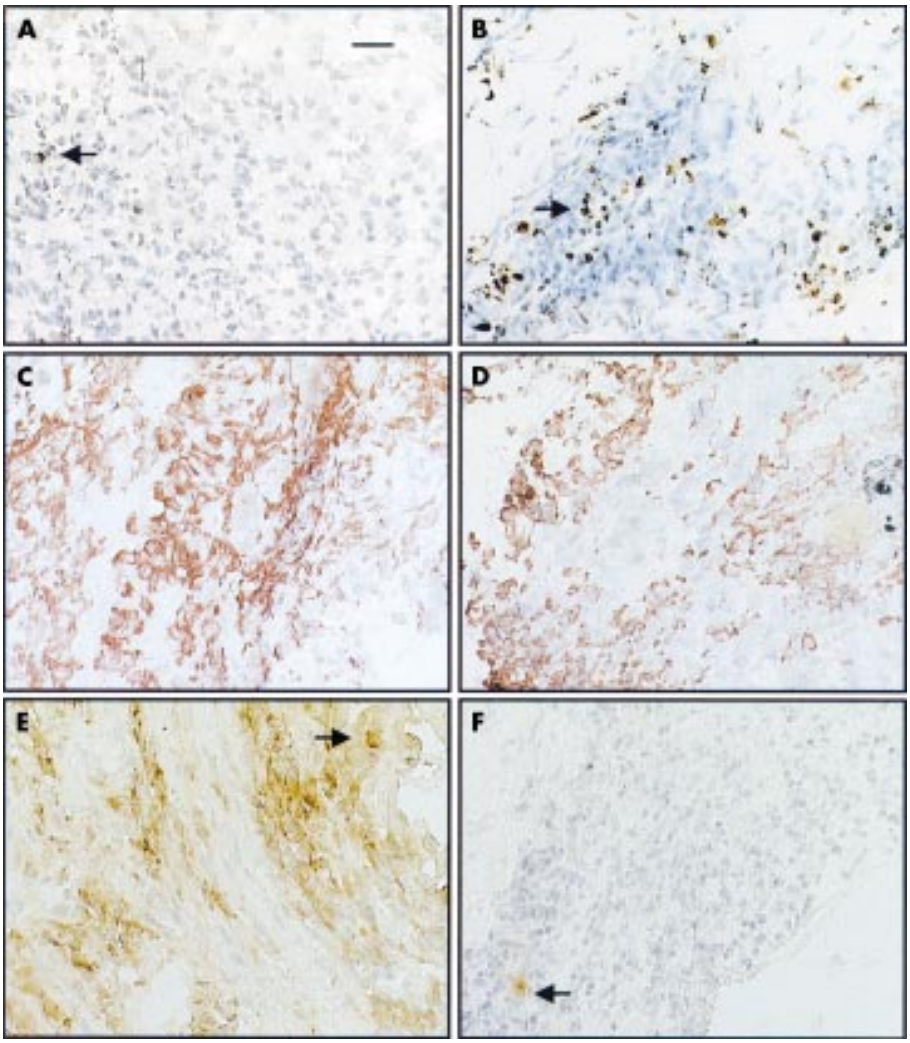

Figure 1 Brown (diaminobenzidine) immunoperoxidase staining for TUNEL, CD68, and FLIP positive cells in serial sections from frozen synovial biopsy specimens counterstained with haematoxylin. (A) TUNEL in early RA (case 1); (B) TUNEL in longstanding RA (case 2); (C) CD68 in early RA (case 1); (D) CD68 in longstanding RA (case 2); (E) FLIP in early RA (case 1); (F) FLIP in longstanding RA (case 2). Original magnification ×340, the bar represent 15 μm.

RESULTS

In early RA few synovial cells were apoptotic (fig 1A). Longstanding RA synovium was characterised by a higher number of apoptotic cells (fig 1B), distributed in clusters, both in lining and sublining layers. CD68 positive cells were detected mainly in early RA (fig 1C) and to a lesser extent in longstanding RA (fig 1D). FLIP positive cells were present in cases of early RA, both in lining and sublining layers, mainly in the macrophage-like cells and to a lesser extent in fibroblast-like synoviocytes (fig 1E). Cases of longstanding RA showed low levels of FLIP positive cells (fig 1F).

Table 1 summarises the results of immunohistochemical analysis of the biopsy specimens. Synovial apoptosis level was increased ($p<0.01$), while macrophage score ($p<0.05$) and FLIP expression ($p<0.01$) were lower in longstanding than in early RA (fig 2). Synovial apoptosis level correlated with CD68 ($R=-0.7$, $p<0.01$) and FLIP expression ($R=-0.53$, $p=0.01$) (data not shown). Disease duration (expressed in months) correlated with synovial FLIP expression ($R=-0.7$, $p<0.001$), apoptosis level ($R=0.6$, $p=0.001$), and CD68 expression ($R=-0.5$, $p<0.05$) (data not shown).

Synovial cytokines and CD3 expression were not significantly different in early RA from longstanding RA (table 1) and did not correlate with apoptosis level. No difference was seen between the eight patients treated with DMARDs and those not treated (11 patients) or between the five patients treated with corticosteroids and those not treated (14 patients).

DISCUSSION

RA is an inflammatory disease characterised by synovial cell accumulation, possibly due to an impaired apoptotic mechanism. Our present data suggest that RA synovial apoptosis is dependent on the disease stage.

Synovial apoptosis evaluated by TUNEL combined with morphological analysis was significantly increased in longstanding RA compared with early cases of RA. TUNEL is the most common method used to detect apoptosis in tissues but may provide false results. However, combination with morphological criteria allows unequivocal identification of dying cells as apoptotic (for review see Stadelmann and Lassmann[11]). We used an image analysis program that allows rejection of false positive staining, permitting combination of the two criteria. Our findings are consistent with a previous report that showed an increase in apoptotic cells in the chronic phase of adjuvant arthritis in rats.[5] Recently, high FLIP expression was detected in synovial biopsy specimens from patients with RA.[7] Interestingly, we found a large variation in FLIP expression between patients. This is in line with the RA synovial heterogeneity previously seen for cytokines[9] and

Table 1 Histological features of synovial tissue from patients with early and longstanding RA. The numbers represent the median values of the percentage of positive cells, evaluated by computer assisted image analysis (for each sample the percentage of positive cells was expressed as percentage of the total cell area evaluated).

Variable	Early RA (n=8)	Longstanding RA (n=11)
TUNEL combined with morphology**	0.6	8.8
Range	0.2–4.65	1.9–29.2
FLIP**	31.1	0.2
Range	0.2–78	0.2–6.8
CD68*	16.2	8.3
Range	6.9–29.8	0.2–14.1
CD3	6.2	3.7
Range	0.5–20.7	0.2–9.2
IL1α	5.8	7
Range	1.3–46.4	0.2–16.4
IL1β	9.7	4.5
Range	0.2–31	0.2–18.2
IL6	0.2	0.2
Range	0.2–7.6	0.2–14.6
TNFα	0.5	0.2
Range	0.2–17.4	0.2–4.97

*p<0.05; **p<0.01.

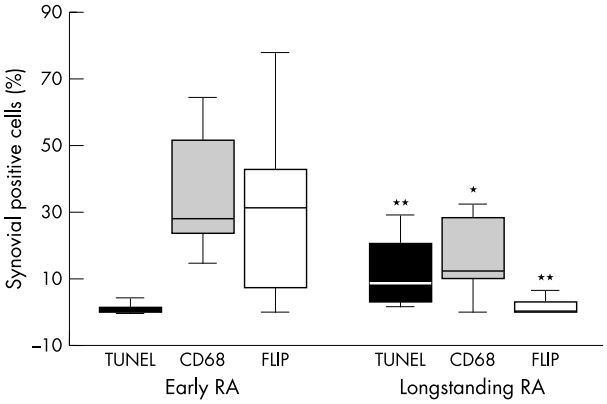

Figure 2 Differences between apoptosis, CD68, and FLIP expression in early compared with longstanding RA. Horizontal lines represent median values and whiskers the non-outliers values. Statistical analysis was performed using the non-parametric Mann-Whitney U test. *p<0.05; **p<0.01.

matrix metalloproteinases.[12] Differences in synovial phenotypes between early and longstanding RA are not due to a general decrease in cellularity as our data are expressed as percentage of total cell area and not total tissue area.

It was beyond the main aim of this study to analyse the effect of different therapeutic strategies owing to the small number of cases included. However, we found no differences between patients treated or not treated with DMARDs or between patients treated or not treated with corticosteroids.

Apoptosis levels in the RA synovium correlate with macrophage score and FLIP expression, suggesting that macrophages are the main cells involved in synovial apoptosis modulation. The decrease in FLIP expression in patients with late disease may either be a primary mechanism responsible for the increased levels of apoptosis or a consequence of increased apoptosis and decreased macrophage number. The influence of disease stage on apoptosis level, CD68 score, and FLIP expression is emphasised not only by the global differences seen between the two groups but also by the direct correlation of each parameter with disease duration.

In conclusion, early RA is characterised by a low level of synovial apoptosis and high macrophage score and FLIP expression. During disease progression the apoptotic mechanism may be restored, suggesting that apoptosis regulation is an important mechanism in determining the long term course of chronic synovitis.

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