Apoptosis in the rheumatoid arthritis synovial membrane: modulation by disease-modifying anti-rheumatic drug treatment

Malcolm D. Smith¹, Helen Weedon², Virginia Papangelis², Jennifer Walker², Peter J. Roberts-Thomson³ and Michael J. Ahern⁴

Abstract

Objectives. RA is characterized at the synovial tissue level by synovial lining hyperplasia, angiogenesis and mononuclear cell infiltrates. A failure of apoptotic pathways may explain these pathological changes in RA synovial tissue. This study aims to demonstrate the presence of initiators and inhibitors of apoptosis in RA synovial tissue and the effect of treatment with DMARDs on apoptotic pathways in RA.

Methods. Synovial biopsy specimens were obtained at arthroscopy from 16 RA patients before and at 3- or 6-month intervals after commencing treatment with a DMARD. Apoptosis (by the terminal deoxy-nucleotidyl transferase mediated dUTP nick end labelling method and polyADP-ribose polymerase staining), proteins regulating apoptosis [Fas, FADD-like IL1b converting enzyme inhibitory protein (FLIP), Bcl-2, Survivin and X-linked inhibitor of apoptosis protein (XIAP)] and the presence of activated caspases (caspases 3 and 8) were detected by immunohistochemistry and quantified using image analysis and semi-quantitative techniques.

Results. Fifteen patients responded to treatment, with an ACR response of ≥20%, 13 achieving an ACR response of ≥50% and 3 achieving an ACR remission. There was a significant reduction in SM macrophages and memory T cells, with an increase in fibroblast-like synovial lining cells following DMARD treatment. Apoptosis was not detected in the inflamed synovial tissue of RA patients before starting treatment, despite evidence of caspase activation, but was detectable after successful treatment with DMARDs. Inhibitors of activated caspases (FLIP, Survivin and XIAP) were detected in RA synovial tissue and were down-modulated with successful DMARD treatment.

Conclusions. Apoptotic pathways are defective in RA synovial tissue from patients with active disease, despite the presence of activated caspases, possibly due to the abundant expression of inhibitors of the caspase pathway in RA synovial tissue. DMARD treatment can modulate apoptosis in the RA SM, which may lead to restoration of the SM architecture towards that of normal synovial tissue.

Key words: Rheumatoid arthritis, Disease-modifying agents, Apoptosis, FLIP, Bcl-2.

Introduction

RA is a chronic inflammatory disorder with characteristic histological features, including synovial lining hyperplasia, angiogenesis and mononuclear cell infiltrates [1]. Hyperplastic changes include both macrophage-like synovial cells and fibroblast-like synovial cells with the fibroblast-like synoviocytes (FLSs) exhibiting pre-neoplastic characteristics, including invasive tendencies and expression of proto-oncogenes [2]. As a result of DMARD treatment, the chronic inflammatory infiltrate in the SM is significantly reduced, returning towards that seen in normal synovial tissue [3, 4]. One explanation for the synovial proliferation is an imbalance between cell proliferation and apoptosis, or programmed cell death and modulation of the apoptotic pathways may explain the changes seen in the SM inflammatory cell infiltrate as a result of DMARD treatment.
Apoptosis is an evolutionarily conserved cell death pathway that occurs in a variety of physiological situations. An apoptotic stimulus induces an initiation and commitment phase, followed by a degradation phase [5]. This last stage is regulated by cysteine proteases (caspases 1–14), which result in apoptosis when activated. There are two central pathways that mediate apoptosis: the Type I ‘extrinsic’ or death receptor pathway generates an apoptotic signal following the aggregation of death ligands and the Type II or ‘intrinsic’ pathway signals through mitochondria [5]. In some cases, Type I activation may also proceed down the mitochondrial pathway [6, 7].

Fas ligand (Fas-L) and TNF-α are classic initiators of the Type I pathway, resulting in the activation of caspase 8, which then activates the effector caspases 3 and 7. There are several inhibitors of the caspase pathway including FADD-like IL1b converting enzyme inhibitory protein (FLIP), X-linked inhibitor of apoptosis protein (XIAP) and Survivin. FLIP suppresses caspase-8 activation by competing with pro-caspases 8 and 10 for binding to Fas-associated protein with death domain [7]. FLIP expression has been demonstrated in RA synovial tissue, predominantly in the lining layer [8, 9]. The Inhibitor of apoptosis protein (IAP) family of proteins are inhibitors of the downstream effector caspases, including activated caspase 3 [10]. A member of the IAP family, Survivin, has been reported to be elevated in serum from RA patients, with high levels correlating with joint erosion in active RA [11]. There is reported synergy between Survivin and XIAP, which can form a complex that promotes increased XIAP stability against ubiquitination and proteasomal destruction [12].

This study has addressed the role of apoptosis in regulating the inflammatory infiltrate in RA synovial tissue, focusing on the effect of successful DMARD treatment on the synovial inflammatory infiltrate and apoptotic pathways.

Materials and methods

Patients

Sixteen RA patients with active synovitis, including an involved knee joint, were recruited for the study. All RA patients fulfilled the ACR criteria for RA [13]. All patients gave informed consent, and the study was approved by the research and ethics committee of the Repatriation General Hospital. Synovial biopsies were taken from the same knee joint before and after commencing treatment with a DMARD at defined intervals (3, 6, 12, 18 and 24 months). The decision about which DMARD was used to treat an individual patient was made by the treating rheumatologist and was not influenced by participation in this study. All patients were followed up at regular intervals (3–6 monthly) for clinical (tender and swollen joint counts, visual analogue scales for pain, patient and physician global assessments and a modified HAQ), laboratory (CRP, ESR and RF) and radiological (X-rays of hands and feet taken annually) parameters. Response to DMARD treatment was assessed by calculating a disease activity score using 28 joints (DAS-28) [14] and ACR response [15].

Synovial tissue

A small-bore arthroscopy (2.7-mm arthroscope, Dyonics, Andover, MA, USA) was performed under local anaesthesia as previously described [16]. At least 10 synovial tissue samples were obtained from all accessible regions of the knee joint, but mainly from the suprapatellar pouch. The samples were processed as formalin-fixed, paraffin-embedded sections and a second set was snap-frozen in Tissue-Tek OCT (Miles Diagnostics, Elkhart, IN, USA) and stored at −80 °C until used. Microtome (4 μm) or cryostat (6 μm) sections were mounted on 3-aminopropyl-triethoxysilane (APTS; Sigma, St Louis, MO, USA) coated glass slides (Superior Marienfeld, Lauda-Königshofen, Germany). The glass slides were boxed and stored at room temperature (paraffin sections) or at −20 °C (frozen sections) until immunohistochemical analysis. Additional synovial tissue from five OA patients and five normal subjects [3] was also stained for apoptosis markers.

Immunohistochemistry

Serial sections were stained with the following mouse mAbs: anti-Fas (Sapphire Bioscience, Crows Nest, New South Wales, Australia), anti-FLIP (all forms of FLIP) (Sigma, St Louis, MI, USA), anti-XIAP and anti-Survivin (R&D Systems, Minneapolis, MN, USA), anti-cleaved caspase 3 (Cell Signaling, Beverley, MA, USA), anti-cleaved caspase 8 (Calbiochem, Darmstadt, Germany), anti-C6D8 (EBM11; Dako Australia, Botany Bay, New South Wales, Australia) to detect macrophages, Mab 67 (Serotec, Oxford, UK), which recognizes CD55, to detect FLSs, anti-FLIP (all forms of FLIP) (Sigma, St Louis, MI, USA), anti-XIAP and anti-Survivin (R&D Systems, Minneapolis, MN, USA), anti-cleaved caspase 3 (Cell Signaling, Beverley, MA, USA), anti-cleaved caspase 8 (Calbiochem, Darmstadt, Germany), anti-C6D8 (EBM11; Dako Australia, Botany Bay, New South Wales, Australia) to detect macrophages, Mab 67 (Serotec, Oxford, UK), which recognizes CD55, to detect FLSs, anti-CD3 (BD Biosciences, San Jose, CA, USA) and anti-CD45Ro to detect T cells and memory T cells, respectively, anti-CD22 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) to detect B cells, anti-CD38 (BD Biosciences) to detect plasma cells and anti-Granyme B (Novo Castra Laboratories, Newcastle upon Tyne, UK). Endogenous peroxidase activity was inhibited using 0.1% sodium azide and 1% hydrogen peroxide in TRIS–phosphate buffered saline (PBS) buffer. Staining for cell markers was performed as described previously [3, 17, 18]. Following a primary step of incubation with mAbs, bound antibody was detected according to a three-step immunoperoxidase method. Horseradish peroxidase (HRP) activity was detected using hydrogen peroxide as substrate and 3-amino-9-ethylcarbazole (AEC) as dye. Slides were counterstained briefly with haematoxylin solution and mounted in Gurr Aquamount (BDH, Poole, UK). Affinity-purified HRP-conjugated goat anti-mouse antibody was obtained from DAKO, affinity-purified HRP-conjugated swine anti-goat Ig from Tago (Burlingame, CA, USA) and AEC from Sigma (St Louis, MO, USA). Controls were included in each histochemical labelling run and included positive control of RA synovial tissue with a known staining pattern and negative
controls including omission of primary antibody and use of isotype-specific irrelevant antibodies. Apoptosis was detected by the terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) method (In Situ Cell Death Detection Kit, POD; Roche Diagnostics, Mannheim, Germany) and confirmed by staining for the p85 fragment of PARP (polyADP-ribose polymerase) (Promega, Madison, WI, USA). Controls for TUNEL staining included DNase treatment of synovial tissue as a positive control and omission of the terminal deoxynucleotidyl transferase enzyme as the negative control, as stated in the TUNEL kit. The confirmation with PARP staining is necessary since TUNEL staining detects DNA breaks that are not specific for apoptosis.

Dual immunohistochemical labelling

Sections of synovial biopsies from 10 RA patients taken at baseline and at various time points after starting DMARD treatment were double labelled for either FLIP, TUNEL or PARP (immunoperoxidase technique) and cell surface markers (CD68, CD55, CD22, CD38, CD3, CD45Ro) (AP technique), using a previously published technique [19]. Additional dual immunohistochemical labelling was performed in the synovial tissue from this subset of patients for activated caspase 3 or 8, and either XIAP or Survivin.

Quantitation of immunohistochemical labelling

After immunohistochemical staining, sections stained for cell surface markers, Fas, FLIP, XIAP, Survivin, Bcl-2, cleaved caspases 3 and 8 (activated caspase 3 and 8), TUNEL and PARP were analysed in a random order by computer-assisted image analysis, using a Videopro system, analysing nine high power fields for each section as previously published [3, 17, 18]. Measurements included mean optical density (MOD), which measures the intensity of staining, area of staining and integrated optical density (IOD), a composite measure of amount and intensity of staining. In addition, these sections were also scored by a semiquantitative method on a five-point scale by two independent observers in a random order, as described previously [20].

Real-time PCR on synovial tissue

cDNAs were prepared from synovial biopsies from five RA patients from the initial synovial biopsy taken before commencing DMARD treatment and again from the biopsy taken when there was significant improvement in disease activity as defined by DAS-28. As controls for these patients, cDNA was prepared from synovial tissue taken at arthroscopic biopsy in five patients with OA of the knee joint. Real-time PCR was performed using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen Life Technologies, Carlsbad, California, USA) as per the manufacturer’s recommendations. Amplification was carried out in a Rotor-Gene 3000 (Corbett Life Science, Mortlake, New South Wales, Australia) with SYBR green detection and melt curve analysis. Oligonucleotide primers used have been described previously, and are specific for caspase-3 [21], Survivin [22] and XIAP [23]. The endogenous reference gene hARP was used to normalize Ct data obtained from the genes investigated. Reaction mixtures contained 10 ng cDNA, Platinum SYBR Green qPCR Supermix-UDG, 300 nM each of forward and reverse primers and diethyl pyrocarbonate (DEPC) treated water to a final volume of 15 μl. All samples were investigated in triplicate and the melting curves obtained after each PCR amplification confirmed the specificity of the SYBR Green assays. Relative expression of the target genes in the studied samples was obtained using the difference in the comparative threshold (ΔΔCt) method [24].

Statistical analysis

Non-parametric statistics were used to analyse the mean ranks of the semiquantitative scores. As the data for area, MOD and IOD, measured by digital image analysis, were not normally distributed, the data were log transformed and analysed using a one-way analysis of variance. Post hoc discrepancies between groups were analysed using Tukey HSD and Bonferroni tests. Statistical significance was accepted when $P < 0.05$. Separate linear regressions of each parameter vs time in months were carried out. To account for the correlation between repeated observations on the same patient in the analyses, generalized estimating equations, assuming an exchangeable correlation structure, were used in the regressions.

Results

Clinical and demographic features

The demographic details of the patients included in the study are shown in Table 1. All patients had active RA at study entry, with the mean DAS-28 score being 6.0 (s.d. 0.6, range 4.4–7.0) and 10 of the 16 patients had a positive RF [mean 279 (333), range 20–1552 IU/ml]. Patients were treated with a range of DMARDs, not including any biological agents, at the discretion of the treating rheumatologist. As a result of the DMARD treatment, 12 patients (75%) were recorded as responders with an ACR of $>50\%$ while 4 patients were recorded as non-responders, with an ACR of $<50\%$. Ten patients achieved a minimum DAS-28 $<2.6$ with DMARD treatment, which equates to low disease activity or remission by the European League Against Rheumatism (EULAR) criteria, while six patients had a minimum DAS-28 $>2.6$ after treatment.

There was a statistically significant difference between responders and non-responders for the lowest levels of CRP (3.18 vs 39.5 mg/l), ESR (13.7 vs 49.7 mm/h), HAQ (0.309 vs 1.317) and DAS-28 (1.17 vs 4.6) ($P < 0.001$ for all measurements) but not for RF (112 vs 170 IU/ml).

Changes in cellular infiltrate in synovial tissue with DMARD treatment

As has been previously described [3], there was a significant change in the cellular infiltration in sequential synovial biopsies from RA patients as a result of DMARD treatment. These changes with treatment were statistically
significant for some [CD68+ macrophages (lining and sublining): \( P = 0.000 \); CD55+ FLSs: \( P = 0.007 \); CD45Ro+ memory T cells: \( P = 0.019 \) but not all (CD22+ B cells, CD38+ plasma cells, CD3+ T cells, Granzyme B+ cells; \( P > 0.05 \) cells involved in the RA synovial membrane inflammatory infiltrate (Fig. 1). There was a direct correlation between a reduction in CRP and DAS-28, and a decrease in CD68+ macrophages in the lining and sublining \( (r = 0.72, P = 0.002 \) for CRP and \( r = 0.58, P = 0.02 \) for DAS-28) and CD45Ro+ memory T cells \( (r = 0.67, P = 0.005 \) for CRP and \( r = 0.63, P = 0.011 \) for DAS-28) and an inverse relationship between CRP and DAS-28 and CD55+ FLSs \( (r = -0.54, P = 0.031 \) for DAS-28).

### Changes in mediators of apoptosis in synovial tissue with DMARD treatment

There were significant reductions in the expression of FLIP (Figs 1 and 2) and Fas (Figs 1 and 3) in the SM of patients who responded to DMARD treatment but not in Bcl-2 expression (Figs 1 and 4). There were significant correlations between changes in DAS-28 and changes in the

#### TABLE 1 Demographics of patients at study entry

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, years</th>
<th>Disease duration, years</th>
<th>Erosions on X-ray</th>
<th>DMARD used</th>
<th>CRP, mg/l</th>
<th>ESR, mm/h</th>
<th>RF, IU/ml</th>
<th>HAQ</th>
<th>DAS-28</th>
<th>No. of synovial biopsies</th>
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<td>RA1</td>
<td>76</td>
<td>0.1</td>
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<td>130</td>
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<td>43</td>
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<td>RA2</td>
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<td>49</td>
<td>141</td>
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<td>5.2</td>
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<td>53</td>
<td>263</td>
<td>2.9</td>
<td>6.4</td>
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<td>RA4</td>
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<td>3</td>
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<td>i.m. Gold + MTX</td>
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<td>120</td>
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<td>2.8</td>
<td>6.8</td>
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<td>4.6</td>
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<td>6.0</td>
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<td>87</td>
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<td>5.2</td>
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<td>i.m. Gold + MTX</td>
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<td>RA15</td>
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<td>0.5</td>
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<td>i.m. Gold</td>
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<td>89</td>
<td>20</td>
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**Fig. 1** Changes in mediators of apoptosis (A) and cellular infiltrate (B) in RA synovial tissue from 16 patients, as a result of DMARD treatment.

Error bars indicate S.D.
Fig. 2 Staining of synovial biopsies from the same knee joint in a RA patient before and after treatment with MTX with an antibody against FLIP (red stain). (A) Baseline biopsy, (B) 3 months, (C) 6 months and (D) 12 months after treatment commenced. Magnification ×200, with inserts ×400.

Fig. 3 Staining of synovial biopsies from the same knee joint in a RA patient before and after treatment with MTX with an antibody against Fas (red stain). (A) Baseline biopsy, (B) 3 months, (C) 6 months and (D) 12 months after treatment commenced. Magnification ×200.
amount of FLIP expression ($r = 0.68, P = 0.004$) but not density of FLIP staining, as well as between changes in DAS-28 and changes in the amount ($r = 0.58, P = 0.024$) and density of staining ($r = 0.52, P = 0.039$) for Fas expression. There were no significant correlations between changes in DAS-28 and Bcl-2 expression in the synovial tissue.

**Effect of DMARD treatment on apoptosis in the SM**

There was little evidence of apoptosis as measured by TUNEL or PARP expression in baseline synovial biopsies from patients with active RA. Although there was evidence of apoptosis as detected by TUNEL (Fig. 5) and PARP (Fig. 6) after DMARD treatment, there was no statistically significant overall change in apoptosis as a result of DMARD treatment, and no correlation between changes in DAS-28 and apoptosis in the synovial tissue of patients responding to DMARD treatment. To confirm that apoptosis pathways had been activated in synovial tissue as detected by TUNEL and PARP staining, a series of sequential synovial biopsies from 10 RA patients taken before and after DMARD treatment were stained for cleaved caspases 3 and 8 and for two inhibitors of the effector caspases, Survivin and XIAP (Fig. 7). This shows that caspases were activated in the synovial tissue from active RA patients but that this did not always lead to the detection of apoptosis in these synovial tissues by the TUNEL method. Successful DMARD treatment (based on DAS-28 < 2.6) resulted in a reduction in SM content of activated caspases 3 ($P < 0.05$) and 8 ($P = 0.024$) as well as the inhibitors of effector caspases, XIAP ($P < 0.05$) and Survivin ($P = 0.014$) (Fig. 7). When OA (Fig. 8) and normal (Fig. 9) synovial tissues were examined for the expression of TUNEL, Fas, caspases 3 and 8, FLIP, XIAP and Survivin, there was no evidence of apoptosis or activated caspases, and there was increased expression of inhibitors of apoptosis (Survivin in OA synovial tissue and FLIP in normal synovial tissue). Real-time PCR results supported the results of immunohistochemistry with reductions (not statistically significant) in caspases 3 and 8 mRNA and significant reductions in Survivin and XIAP mRNA (Table 2) in synovial tissue with successful DMARD treatment ($P < 0.05$).

Double labelling for TUNEL and cell lineage-specific markers demonstrated apoptosis, mainly in synovial lining macrophages with no detectable apoptosis in B cells, plasma cells, synovial lining fibroblasts or T cells (results not shown). Similar results were seen for PARP staining (results not shown). Double labelling with FLIP and cell surface markers demonstrated that the majority of FLIP staining was seen in CD68$^+$ macrophages (Fig. 10). Double labelling for activated caspases 3 and 8 and Survivin and XIAP demonstrated that synovial tissue cells expressing activated caspases also expressed a significant amount of either XIAP or Survivin, as we have recently published [25].
Comparison between DMARD responders and non-responders

There were significant differences between responders and non-responders to DMARD treatment for FLIP ($P = 0.009$), FAS ($P = 0.017$) and CD68 ($P = 0.022$), all of which were significantly lower in responders compared with non-responders. There was also a significant difference in the area of CD55$^+$ lining fibroblasts, which were increased in responders over non-responders ($P = 0.001$). These differences were seen regardless of whether response to DMARD treatment was based on an ACR response $>50\%$ or a DAS-28 $<2.6$.

**Discussion**

In RA, Fas and Fas-L have been detected in synovial cells and, more controversially, in activated mature T cells obtained at the time of arthroplasty. These cells have been shown to be susceptible to Fas-mediated apoptosis induced by an anti-Fas mAb, and this sensitivity has been limited to RA cells when directly compared with OA controls [26–31]. The inflammatory milieu of the rheumatoid cells is likely to contribute to the degree of Fas-mediated apoptosis. TNF-$\alpha$ and IL-1 have been demonstrated to suppress apoptosis $in$ $vitro$ and TNF-$\alpha$ has also been demonstrated to induce susceptibility to Fas-mediated apoptosis [32, 33]. We have previously demonstrated, in a similar patient population to that used in this study, that DMARD treatment will successfully down-modulate the SM content of inflammatory cytokines [34] as well as restore the SM architecture back towards that observed in OA and even normal synovial tissue [3, 4]. Obviously, other factors must influence the effects of these key cytokines. Downstream signalling of Fas is also likely to influence cellular susceptibility to apoptosis as evidenced by the fact that OA synoviocytes seem resistant to the

**Fig. 5** Staining of synovial biopsies from the same knee joint in a RA patient before and after treatment with MTX. (A–C) Baseline biopsy, (D–F) 3 months, (G–I) 6 months and (J–L) 12 months after treatment commenced. Staining for cleaved caspase 3 (A, D, G and J), cleaved caspase 8 (B, E, H and K) and TUNEL (C, F, I and L). Magnification $\times$200.
Fig. 6 Staining of synovial biopsies from the same knee joint in a RA patient before and after treatment with MTX with an antibody against PARP (brown stain). (A) Baseline biopsy, (B) 3 months, (C) 6 months and (D) 12 months after treatment commenced. (E) OA synovial tissue stained for PARP. (F) OA synovial tissue negative control. (G) Normal synovial tissue stained for PARP. (H) Normal synovial tissue negative control.

Magnification ×200.
Apoptosis pathways are defective in RA synovial tissue from patients with active disease, possibly due to the abundant expression of inhibitors of the caspase pathway in RA synovial tissue. DMARD treatment can modulate apoptosis in the RA SM. A future strategy to target therapies in RA could be aimed at modulating the proteins that regulate apoptotic pathways in the RA synovial tissue. FLIP, XIAP and Survivin are logical initial targets, given that standard DMARD treatments can successfully reduce the expression of these inhibitors in RA synovial tissue and reduce the chronic inflammatory synovial infiltrate, at least in part through activation of apoptotic pathways.

**Rheumatology Key messages**

- Apoptosis pathways are defective in active RA synovial tissue due to inhibitors of apoptosis.
- Successful DMARD treatment of RA can restore apoptosis in synovial tissue by reducing apoptotic pathway inhibition.
- FLIP, XIAP and Survivin are potential therapeutic targets in RA.
Fig. 8 OA synovial tissue stained for apoptosis markers. (A) DNase-treated positive control, (B) TUNEL, (C) Fas, (D) caspase 3, (E) caspase 8, (F) FLIP, (G) XIAP and (H) Survivin.

Magnification ×200.
Fig. 9 Normal synovial tissue stained for apoptosis markers. (A) DNase-treated positive control, (B) TUNEL, (C) Fas, (D) caspase 3, (E) caspase 8, (F) FLIP, (G) XIAP and (H) Survivin.

Magnification ×200.
Table 2 Results of real-time PCR on RA synovial tissue, expressed as an averaged Ct with ranges in parentheses

<table>
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<tr>
<th>Protein</th>
<th>RA synovial tissue at baseline</th>
<th>RA synovial tissue after DMARD treatment</th>
<th>OA synovial tissue</th>
</tr>
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<tbody>
<tr>
<td>Caspase 3</td>
<td>24.92 (22.51–30.12)</td>
<td>27.24 (24.35–29.88)</td>
<td>26.01 (25.02–29.49)</td>
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</table>

Figure 10 Single labelling for CD68 using an AP method with fast blue as the chromogen (A and B). Double labelling for CD55 (blue) and FLIP (immunoperoxidase method, AEC as chromogen shown as red staining) (C and D) or CD68 (blue) and FLIP (red) (E and F) with double labelling shown as purple staining.

(A, C and E) magnification ×200. (B, D and F) magnification ×400.
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References


