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ORIGINAL ARTICLE

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Bcl-x_L expression in vivo in rheumatoid synovium

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Abstract To examine the expression of the apoptosis regulatory protein, Bcl-x_L, in the synovium of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). Immunohistochemistry for Bcl-x_L was carried out on synovial samples from patients with RA and OA. Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis were performed to qualitatively examine the expression of Bcl-x_L. Bcl-x_L expression was detected in the lining, endothelium and inflammatory cells of both RA (n=20) and OA (n=10) samples. However, there was significantly more expression in the lining of RA synovium compared to OA (77 vs 61%, p<0.05). Many of the positive cells in the RA subsynovium were noted to be plasma cells. There was a significant correlation between Bcl-x_L expression and the number of inflammatory cells in the subsynovium of RA and OA patients (r_s=0.376, p<0.05, n=30). Age and disease duration did not correlate with Bcl-x_L expression in rheumatoid patients. Bcl-x_L may play a role in the extended survival of synoviocytes and inflammatory cells in rheumatoid synovium.

Keywords Apoptosis · Rheumatoid arthritis · Synovium

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Introduction

Rheumatoid arthritis (RA) is an autoimmune condition characterised by a florid inflammatory infiltrate of the synovial tissue in response to an unknown antigen [1]. The pathological hallmark of the disease is synovial hyperplasia with infiltration by inflammatory cells, ultimately leading to invasion of local cartilage and bone by synovial overgrowth (pannus) [2]. Apoptosis is a means of 'programmed cell death', which is necessary in many physiological processes; for example, the elimination of immunocytes at the end of immune responses [3, 4]. Dysregulation of apoptosis has been implicated in many disease processes, in particular, cancer and autoimmunity [5]. In RA, there is excessive proliferation of synoviocytes as well as accumulation of inflammatory cells. Fas-mediated apoptosis has been shown to occur in rheumatoid synovial cells [6]. However, the relative sparing of lymphocytes from apoptosis in RA synovium [7] suggests that the control of the apoptotic pathway may be dysfunctional.

Members of the Bcl-2 family regulate apoptosis by altering mitochondrial permeability and the release of apaf-1 and cytochrome c [8, 9]. It is postulated that the control of apoptosis depends on the ratio of pro- and anti-apoptotic Bcl-2 proteins [10]. Bcl-2 is overexpressed in RA synovial tissue compared to osteoarthritis (OA), and Bcl-2 expression has also been shown to be critical in mitochondrial homeostasis and cell survival in RA synovial fibroblasts [11]. Bcl-x_L is an anti-apoptotic homologue of Bcl-2 [12] and has been shown to enhance the survival of immature B

cells in transgenic mice [13]. Human peripheral blood memory B cells have been found to co-express high levels of Bcl-2 and Bcl-x_L, and this may contribute to B-cell survival [14]. There is also evidence that Bcl-x_L plays a role in the protection of synovial cells from apoptosis [15]. Human peripheral B cells were found to up-regulate expression of Bcl-x_L when co-cultured with a stromal cell line derived from RA synovium and, consequently, were protected from apoptosis. This protection of B cells from apoptosis was blocked by the use of antibodies to vascular cell adhesion molecule 1 (VCAM-1), which is expressed on the stromal cells. Hence, signals from stromal cells appear to regulate B-cell apoptosis in the microenvironment of the rheumatoid joint. CD40-activated human B cells have also been found to up-regulate Bcl-x_L expression, protecting the cells from Fas-mediated apoptosis [16]. More recent work has demonstrated that Bcl-x_L also plays a part in the survival of activated macrophages in inflammatory conditions [17].

The aim of this study is to compare the expression of Bcl-x_L in RA and OA synovium in vivo, and to determine the relationship, if any, between Bcl-x_L expression and other variables such as patient age and disease duration.

Materials and methods

Tissues

Synovial tissue was obtained from RA (n=20) and OA (n=10) patients who were undergoing elective joint replacement, after a protocol approved by the Ethics Committee of Cork University Hospital. Informed consent was obtained and patients fulfilled American College of Rheumatology criteria. Synovium for reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analysis was flash-frozen in liquid nitrogen and stored at −80°C. Synovium for immunohistochemistry was placed in formaldehyde before processing. Sixteen of 17 patients in the RA group were seropositive (rheumatoid factor status not recorded in three cases). The median disease duration was 11 years (range 6 months–40 years), and the median number of DMARDs used was 2 (range 0–4).

Histology and immunohistochemistry

Using a microtome, 5-μm-thick sections were cut from blocks of paraffin-embedded synovium. Sections were deparaffinised in xylene for 10 min and were rehydrated in an alcohol gradient. Antigen retrieval was by microwave irradiation at 750 W in citrate buffer, pH 6.0, until it boiled and then at 100 W for 15 min. Sections were immunostained with a Bcl-x_L-specific rabbit polyclonal antibody (Oncogene, CN Biosciences, Nottingham, UK) at a dilution of 1:1,000, followed by incubation with a biotinylated goat anti-rabbit secondary antibody (Vector, Burlingame, CA, USA). Slides were then incubated with an avidin–biotin–peroxidase complex (Vector) for 30 min at room temperature. Antibody binding was localised using diaminobenzidine and sections were counterstained with Mayer's haematoxylin (Sigma, St. Louis. MO, USA). Negative controls were performed by omitting the primary antibody. Consecutive sections were stained for plasma cells with a monoclonal antibody specific for CD138 at a 1:1,000 dilution.

The immunostained sections were examined to determine the distribution of Bcl-x_L staining. Samples were initially examined to quantify the degree of lining hyperplasia. To examine the relationship between Bcl-x_L expression and synovial inflammation, an inflammatory score (0–5) was assigned to each sample on the basis of the number of inflammatory infiltrates seen in each biopsy specimen under ×10 magnification. Tissue characteristics and inflammatory scores are shown in Table 1. The number of positive-staining cells in the synovial lining was quantified for both RA and OA and expressed as a percentage of the total lining cells. Subsynovial expression of Bcl-x_L was graded using a modified Lindblad–Hedfors scoring system [18].

Reverse transcriptase polymerase chain reaction

Bcl-x_L mRNA was detected using RT-PCR. RT-PCR for the housekeeping gene β-actin was performed as a positive control. RNA was extracted using an RNA extraction kit (Qiagen, West Sussex, UK). Complementary DNA (cDNA) was synthesised using random hexamer primers and AMV reverse transcriptase (both Promega, Madison, WI, USA). PCR was performed using the following sense and antisense primers: Bcl-x_L—ACAGCCCCGCG GTGAATGG and TGCCCCGCCGAAGGAGAAA, and β-actin—GTGGGGCGCCCCAGGCACCA and CTCC TTAATGTCACGCACGATTTC. The PCR products for Bcl-x_L and β-actin were 593 and 560 bp, respectively. Thermal cycling was as follows: denaturation at 96°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 3 min. Thirty-two cycles were performed for the Bcl-x_L PCR and 35 cycles for the β-actin control. Primers were used at a final concentration of 0.1 μM, deoxynucleotide tri-phosphates at 50 μM and $MgCl_2$ at 1.5 mM. One unit of Taq DNA polymerase was used per 50 μl of reaction

Table 1 Bcl-x_L positivity

	RA (n=20)	OA (n=10)
Age (median in years)	54.5	68.0
Percent of females	60	40
Lining hyperplasia	3.2 (2.7–3.7)	1.9 (1.5–2.3)
Inflammatory score	2.8 (1.9–3.6)	1.5 (0.3–2.7)
Synovial lining staining (%)	77 (65–88)	61 (39–82)
Subsynovial staining		
Grade I (1–15%)	1	0
Grade II (16–50%)	7	4
Grade III (51–85%)	9	5
Grade IV (>85%)	3	1

mixture. PCR products were resolved by electrophoresis on a 2% agarose gel and viewed under ultraviolet illumination after staining with ethidium bromide. A 100-bp DNA size ladder (Promega) was used to establish product size.

Western blot analysis

Frozen synovial tissue was placed in a lysis buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 1% Nonidet P-40, 0.5 mM phenylmethyl sulphonyl fluoride, 0.25% sodium dodecyl sulphate, 0.5 mM dithriothreitol, 1 μg/ml leupeptin, 10 u/ml aprotinin and 10 μg/μl of soyabean trypsin inhibitor, and was then immediately homogenised with a probe sonicator. Samples were centrifuged to remove particulate debris. Protein concentrations of the lysates were determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Equivalent amounts of protein for each specimen were electrophoresed through a 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel and transferred by a semi-dry method (Hoefer SemiPhor, Pharmacia Biotech, San Francisco, CA, USA) onto a nitrocellulose membrane. The membranes were immersed in blocking buffer (5% dried milk to 0.1% TBS-Tween) overnight at 4°C and were then incubated with a Bcl-x_L-specific goat polyclonal IgG antibody (Santa Cruz Biotechnology, CA) at a dilution of 1:100 for 3 h at room temperature. Washed membranes were incubated with a horseradish peroxidase-conjugated secondary anti-goat IgG antibody (1:2,000, Dako, Carpenteria, CA, USA). Washing between incubations was carried out using 0.1% TBS-Tween. Immunoreactive products were detected using chemiluminescence reagent (Amersham Pharmacia Biotech, Buckinghamshire, UK) and exposed to autoradiography film. Pre-stained low range molecular weight sodium dodecyl sulphate-polyacrylamide gel electrophoresis standards were used to determine protein size (Bio-rad, Hercules, CA, USA).

Statistical analysis

Differences between the RA and OA groups were examined for statistical significance using the Mann–Whitney two-sample test. Correlations were determined using Spearman's correlation coefficient (r_s). $p<0.05$ was considered to be significant.

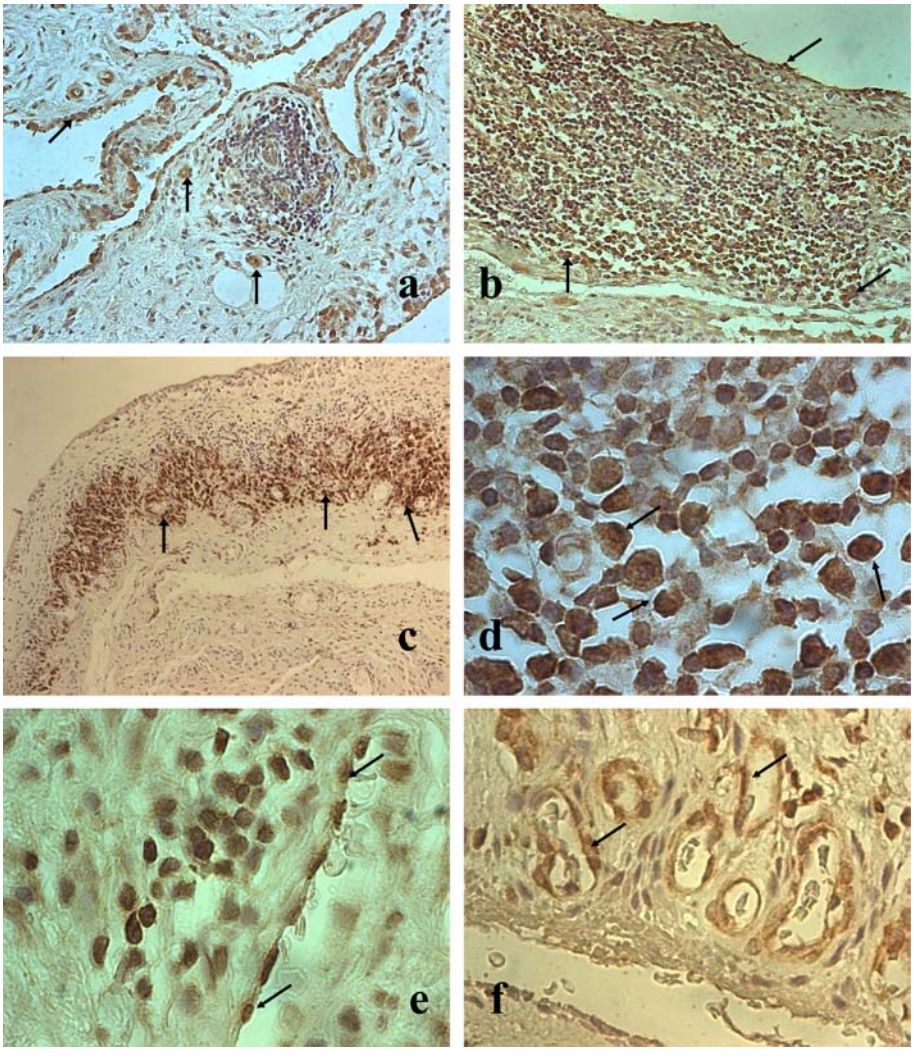

Fig. 1 Bcl-x_L expression in OA and RA synovium. Sections of synovium were stained with a Bcl-x_L-specific polyclonal antibody at a dilution of 1:1,000. Positively stained cells are brown (*arrowed*). Negative controls were performed by omitting the primary antibody (not shown). **a** and **b** Bcl-x_L staining in OA and RA (×20). **c** and **d** Expression of Bcl-x_L in plasma cells in RA synovium. CD138 staining for plasma cells in RA synovium is shown in **c** (×10) with Bcl-x_L staining in a consecutive section in **d** (×100). **e** and **f** Endothelial staining for Bcl-x_L in RA and OA synovium (×100 and ×63, respectively)

Results

Bcl-x_L was expressed in the synovial lining of both RA and OA, but there was significantly more Bcl-x_L expression in the rheumatoid synovial lining. There was no significant difference, however, in the degree of Bcl-x_L expression between the subsynovium in RA samples and that in OA samples. There was a significant correlation between the subsynovial expression of Bcl-x_L and the number of inflammatory cells in the subsynovium (r_s=0.376, p< 0.05). Age and disease duration did not correlate with Bcl-x_L expression. Subsynovial Bcl-x_L was found predominantly in the mononuclear inflammatory infiltrates. Histological examination (×100) showed many of these positive cells to be plasma cells. CD138 staining for plasma cells on consecutive sections confirmed that plasma cells accounted for much of the positive Bcl-x_L staining seen in the RA samples (Fig. 1). Expression of Bcl-x_L was also noted in endothelial cells in both RA and OA samples (Fig. 1).

Expression of Bcl-x_L mRNA in synovial samples from RA (n=7) and OA (n=7) patients was analysed using RT-PCR. Bcl-x_L mRNA was expressed in all synovial samples, and there was no significant difference in the level of Bcl-x_L mRNA expression between specimens derived from patients with RA and OA (Fig. 2). Western blot analysis was performed to qualitatively assess the expression of Bcl-x_L protein in both RA and OA. Western blotting revealed significant expression of Bcl-x_L at the protein level in three out of four RA samples, with no Bcl-x_L protein detected in the three OA specimens at equivalent protein loading concentrations (Fig. 3).

Discussion

Defects in apoptosis in RA have been detected in the signalling molecules (Fas, TNF and p53) [19, 20] and also in the regulatory pathways; for example, the Bcl-2 family [20]. Increased Bcl-2 expression in RA synovial fibroblasts, for example, is associated with reduced apoptosis and enhanced fibroblast survival [21]. Fas and FasL are expressed in synoviocytes, monocytes and lymphocytes in rheumatoid synovium; however, despite coexpression of Fas and FasL, apoptosis is detected in only 1% of these cells in RA [21].

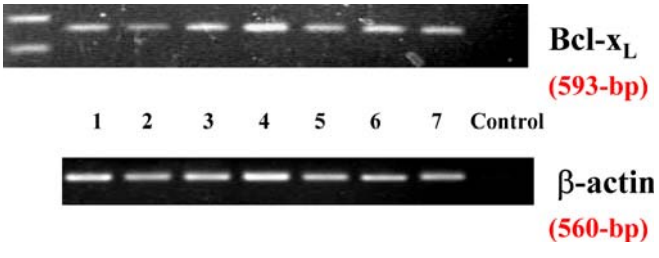

Fig. 2 RT-PCR for Bcl-x_L mRNA expression in RA and OA synovium. Bcl-x_L mRNA was expressed in both RA (lanes 1, 3, 5 and 7) and OA (lanes 2, 4 and 6) samples

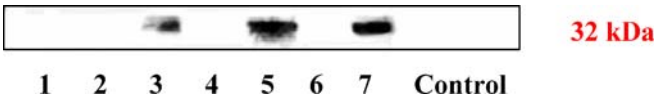

Fig. 3 Western blot analysis for Bcl-x_L. Immunoblotting was carried out using a Bcl-x_L-specific IgG antibody. Bcl-x_L expression was demonstrated in three out of the four RA samples (lanes 1, 3, 5 and 7), but there was no detectable Bcl-x_L expression in the three OA samples at equivalent protein concentrations (lanes 2, 4 and 6)

In the present study, Bcl-x_L expression was detected in RA and OA in the synovial lining and stroma, and also in inflammatory cells and endothelial cells, using immunohistochemistry. There was significantly more Bcl-x_L protein expressed in the synovial lining in the RA samples compared to OA synovium. There was also a significant correlation between the expression of subsynovial Bcl-x_L and the number of inflammatory cells in the subsynovium. Disease duration or patient age did not correlate with the amount of Bcl-x_L expressed. Qualitative evaluation using Western blotting showed increased Bcl-x_L protein expression in the RA samples relative to OA controls (Fig. 3), which correlates with the higher inflammatory score for the RA samples. Immunohistochemistry for CD138 revealed that a large proportion of the Bcl-x_L-positive cells were plasma cells (Fig. 2).

Evidence from in vitro work supports the hypothesis that Bcl-x_L contributes to the survival of cells in RA synovium. Stromal cells isolated from RA synovium have been shown to protect co-cultured B cells from cell death by up-regulating Bcl-x_L expression in the B cells [15]. This up-regulation of Bcl-x_L appears to be mediated by the expression of VCAM-1 on the stromal cells, which cross-links with VLA-4 (very late antigen 4) on the B cells. It has been shown that synoviocytes cultured in vitro support the survival of co-cultured B cells and also induce the terminal differentiation of B cells into plasma cells, with high levels of intracytoplasmic immunoglobulins [22]. Up to 2% of synovial plasma cells in RA are bilobed [23], suggesting local proliferation of plasma cells; there is also evidence that plasma cells have a prolonged survival time in rheumatoid synovium [24]. In this study, many of the Bcl-x_L-expressing cells were noted to be plasma cells morphologically and by staining of consecutive sections with an antibody for the plasma cell marker CD138. Up-regulation of Bcl-x_L may be a mechanism by which plasma cell survival is prolonged in rheumatoid synovium.

Plasma cells in the synovial fluid of patients with seropositive RA have been found to produce IgM rheumatoid factor (RF), and the microenvironment in the joint is thought to favour RF production [25]. Although the precise role of RF in the aetiology of RA is not clear, there is an association between high-titre RF and disease severity [26, 27]. As well as producing autoantibodies, IgG-producing plasma cells have been found to secrete TNF-α in chronic diseases such as rheumatoid arthritis and polyarteritis nodosa [28]. Bcl-x_L is up-regulated in malignant plasma cells in patients with myeloma [29], and a similar mechanism may account for the abnormal persistence of plasma cells in rheumatoid synovium.

Synovial fluid T cells have been found to up-regulate the expression of Bcl-x_L, possibly contributing to their longevity in synovial fluid. It has also been found that the pro-apoptotic protein Bax is overexpressed relative to Bcl-x_L in the synovium of patients with numerous apoptotic cells; however, this was not the case in synovium with few apoptotic cells, as detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling technique [30]. A further study found marked colocalization of Bax and Bcl-x_L in RA synovium which was associated with a low rate of apoptotic cells, again suggesting that the ratio of Bcl-2 homologues is important in RA synovium [31].

In conclusion, our results show that RA is associated with elevated expression of the anti-apoptotic protein, Bcl-x_L, in the synovial lining and also in inflammatory cells, particularly plasma cells. Increased expression of Bcl-x_L may contribute to the survival and proliferation of inflammatory cells, including plasma cells, in rheumatoid synovium, and Bcl-x_L may be a potential therapeutic target in RA.

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