

Changes in Apoptotic Gene Expression in Lymphocytes from Rheumatoid Arthritis and Systemic Lupus Erythematosus Patients Compared with Healthy Lymphocytes

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Abstract

Introduction Systemic lupus erythematosus (SLE) and rheumatoid arthritis have complex genetic traits, but in both autoimmune diseases, dysfunctional apoptosis appears to play a part in disease pathology. This study examined the levels of in vitro apoptosis in lymphocytes from healthy, rheumatoid arthritis (RA) and SLE individuals and related observed differences to their lymphocyte apoptosis gene profiles.

Materials and Methods Lymphocytes were assessed for cell death by nuclear pyknosis and DNA fragmentation. Control, SLE and RA apoptosis gene profiles were obtained by quantitative real-time polymerase chain reaction (QRT-PCR) analysis.

Results and Discussion The mean levels of pyknosis in RA and SLE freshly isolated lymphocytes were significantly higher than in control lymphocytes. Ninety-three apoptosis genes were analysed by QRT-PCR of mRNA from RA, SLE and healthy lymphocytes. We identified significant differences ($p < 0.05$) in the expression of the same 11 of 93 and two of 93 apoptotic genes in individual SLE and RA patients tested as compared with controls.

Conclusion We propose that similarly altered expression of specific apoptotic regulatory genes (e.g., the death effector domain-containing DNA-binding protein and apoptosis-associated speck-like protein containing a CARD) occurs in the lymphocytes of individual patients

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with SLE or RA that may influence the extent and rate of spontaneous apoptosis in these autoimmune conditions.

Keywords *DEDD2* up-regulation · *PYCARD* down-regulation · pyknosis · real-time PCR · lymphocytes · lupus · rheumatoid arthritis

Introduction

It has been suggested that defective apoptosis (programmed cell death) may play a key role in human autoimmune diseases by causing an imbalance between the production and destruction of various cell types including lymphocytes [1–3]. It appears that the main mechanism by which “aged”/mature T cells are removed from the circulation involves apoptosis [4–6]. Identification of the molecular events leading to leukocyte apoptosis has been the subject of considerable attention [7, 8], and the persistence of these cells might be caused by dysregulation of apoptosis. Indeed, many laboratories have implicated several products of signalling pathways in defective apoptosis including FasL, NF- κ B [9] and Bcl-2 [10]. This could ultimately lead to B and T cell hyperactivity resulting in excessive accumulation of autoantigens, polyclonal B cell activation and increases in autoantibody production [5, 11, 12]. Patients with autoimmune disease are treated with potent inducers of apoptosis (e.g. prednisolone and methotrexate), but the apoptotic genes that are influenced by these medications are not clearly established [13–15].

Lymphocytes are instrumental in regulating apoptosis of other cell types. For example, by cytotoxic T cells through the release of proteases such as perforin and granzyme B, and by T lymphocyte-derived Fas ligand (FasL/CD95L) which interacts with Fas (Apo-1/CD95) on epithelial cells. Moreover, an imbalance between the down-regulated apoptosis-inhibitor Bcl-2 and the up-regulated apoptosis-inducer Bax, or the autocrine and/or paracrine Fas/FasL interaction may influence the signals for survival or death of lymphocytes themselves or cells which they have identified for removal. Lymphocytes infiltrating the salivary glands of lupus-prone mice [16] and synovial fluid of RA patients [17] are blocked in their ability to commit to apoptosis. Alterations in the expression of the apoptosis-inducer Fas or expression of Bcl-2 may explain the resistance of these cells to apoptosis, resulting in a prolonged production of proinflammatory cytokines and autoantibodies. This could underlie the chronic inflammation at selected tissue sites in various autoimmune diseases. Furthermore, circulating lymphocytes from patients (systemic lupus erythematosus (SLE), rheumatoid arthritis and myositis) have increased levels of constitutive DNA damage, show reduced DNA repair capacity and increased

susceptibility to killing by genotoxic agents [18]. We have previously shown increased levels of oxidative DNA damage in peripheral blood mononuclear cells from patients with RA and SLE suggesting an increased population of “pre-apoptotic” cells [19]. Moreover, post-translational modification of cell surface apoptotic recognition molecules may influence the sensing and removal of apoptotic cells in autoimmune conditions [20].

Pyknosis is a valid indicator of lymphocyte death and is the key “gold standard” morphologic method of distinguishing apoptotic cells [21–23]. We therefore examined the possibility that dying lymphocytes accumulate in human autoimmune diseases by comparing the levels of in vitro apoptosis in lymphocytes from patients with RA, SLE and controls by morphological (light microscopy) examination of individual lymphocytes on slides (slide-death method) and by examining the cellular DNA for the characteristic 180 base-pair fragmentation. Apoptotic regulation has been the focus of attention in several autoimmune diseases: In SLE, there is accumulation of apoptotic debris, possibly resulting from excessive cell death at a rate greater than can be cleared [24, 25]. Similarly, proinflammatory T lymphocytes appear to be resistant to apoptosis in the RA synovium [17, 26, 27]. In this study, we used QRT-PCR to quantify apoptotic gene expression in cells from controls and patients with autoimmune disease. We demonstrate that distinct differences in cellular apoptosis are observed in SLE, RA and normal individuals within each subject group, revealing distinct apoptotic expression patterns in autoimmune patients at the cellular level and mRNA level.

Methods

Patients and Controls, Cell Isolation and Culture

For the pyknosis, DNA fragmentation and QRT-PCR assays, 14 patients with RA, 10 patients with SLE and 11 healthy volunteers were enrolled. For Q-PCR analysis, five RA patients (all on methotrexate), seven SLE patients (six on at least prednisolone; see Table 1 for other medication) and eight healthy age-matched control subjects were selected. All RA patients fulfilled the 1987 American College of Rheumatology (ACR) criteria for RA [28], and SLE patients fulfilled the 1982 ACR criteria for SLE [29]. Informed consent was obtained from all patients (MREC project number 06/Q2102/56). Due to the relatively large volumes of blood required for both pyknosis and QRT-PCR replicate analysis, it was not possible to perform both analyses on the same patients. In the genomic studies, disease activity in the patients with SLE was assessed using the British Isles Lupus Assessment Group (BILAG) index [30]. For the purposes of this study, the BILAG grades were

Table 1 Demographic Characteristics, Disease Activity, Medication and Age of the SLE and RA Patients in the QRT-PCR Analysis

Disease [sample no.]	Sex	Age	Disease activity ^a	Medication
SLE [78]	F	30	BILAG 1	Prednisolone, mycophenolate mofetil
SLE [71]	F	42	BILAG 4	Prednisolone, azathioprine
SLE [30]	F	31	BILAG 0	Hydroxychloroquine
SLE [22]	F	42	BILAG 3	Prednisolone, hydroxychloroquine
SLE [25]	F	40	BILAG 4	Prednisolone
SLE [66]	F	34	BILAG 0	Prednisolone, azathioprine, hydroxychloroquine
SLE [79]	F	38	BILAG 0	Prednisolone, azathioprine
RA [46]	F	44	CRP 41 mg/L DAS28 _{CRP} =4.37	Methotrexate, Hydroxychloroquine
RA [56]	M	72	CRP 18 mg/L DAS28 _{CRP} =3.11	Methotrexate
RA [47]	F	64	CRP 22 mg/L DAS28 _{CRP} =3.89	Methotrexate, sulfasalazine
RA [91]	M	58	CRP 12 mg/L DAS28 _{CRP} =3.52	Methotrexate
RA [66]	F	38	CRP 3 mg/L DAS28 _{CRP} =3.68	Methotrexate

^a SLE disease activity was assessed using The British Isles Lupus Assessment Group (BILAG) grades, which were converted into global index using the following notation: A=9 points, B=3 points, C=1 point, and D/E=0, with arbitrary cut-off values of 0–5 for inactive disease and ≥ 6 points for active disease. RA disease activity was assessed by DAS28. SLE patients (seven females, mean age 36.7, range 30–42), RA patients (three females and two males, mean age 55.2, range 38–72), healthy volunteer controls (six females and one male, mean age 35.2 years, range 20–43). Cutoffs for Levels of Disease Activity Defined by DAS28_{CRP}>3.7 high; 2.4–3.7 moderate; 1.6–2.4 low; <1.6 remission [52]

converted into a global index where grade A=9 points and grade D/E=0 points. Arbitrary cut off values of 0–5 points for inactive disease and ≥ 6 points for active disease were used to distinguish the level of disease activity in the patients. Disease activity of RA patients was assessed by swollen joint counts, C-reactive protein (CRP) and 28 joint disease activity score (DAS28_{CRP}).

Peripheral blood samples were obtained from all patients and healthy volunteers in parallel. Lymphocytes from subjects were isolated using standard lymphoprep™ [31]. Cells from each sample were examined immediately (0 h) for nuclear pyknosis (see below) or cultured at a concentration of 1×10^6 cells/ml in culture medium (RPMI-1640) with 20% autologous serum at 37°C in a 5% CO₂ in air for 24 h.

Analysis of Apoptosis

Apoptotic cell death can be measured by numerous morphological and biochemical methods. The most common techniques employed are terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labelling (TUNEL) staining, Annexin V binding and DNA laddering, but are vulnerable to generating false positive results [32, 33]. This emphasises the importance of verifying results by at least two independent methods and preferably one method that looks at the cells directly. In our study, we employed a DNA fragmentation assay together with a pyknotic lympho-

cyte assay since the morphological changes occurring in cells is what is actually observed in SLE patients.

Measurement of pyknotic cells by light microscopy: Slides of intact lymphocytes were prepared with Susa fixative and stained with Mayer's hemalum with subsequent dehydration in 2-ethoxy ethanol (Cellosolv), and 500 cells on each slide were examined. Cells exhibiting nuclear pyknosis and optically homogeneous nuclear chromatin were counted as histologically dead (Fig. 1b) and were readily distinguishable from surviving counterparts. Cells were analysed at 0 h and after 24 h in culture by blinded observers. For the DNA fragmentation assay, DNA was prepared by standard procedures from approximately 5×10^6 lymphocytes. Samples were treated with 100 μ g/ml proteinase K and 20 μ g/ml DNase-free RNase at 56°C for 30 min and run on a 1.5% w/v agarose gel. After agarose electrophoresis, gels were stained with ethidium bromide, exposed to ultraviolet (UV) light and photographed.

RNA Isolation and cDNA Preparation for Apoptotic Gene QRT-PCR Analysis

For the QRT-PCR studies, 40–50 ml of blood was obtained per subject, from which fresh RNA was isolated from 5×10^6 purified lymphocytes from SLE, RA and sex-matched control subjects using the RNeasy mini kit and the RNA supplemented with 10 μ l RNA stabilisation reagent

(Quiagen® Ltd, West Sussex, UK). For the QRT-PCR Taqman apoptotic tiled low density (TLDA) gene array (Applied Biosystems, Warrington, UK, part no. 4378701) analysis, complementary DNA (cDNA) libraries were generated from up to 2 µg of total RNA from individual patients and healthy controls, employing a high-capacity reverse transcription kit and random primers (Applied Biosystems) in accordance to the manufacturer's instructions.

QRT-PCR of Apoptotic Genes in SLE, RA and Healthy Individuals

Although this technique has not been previously used to analyse differences in mRNA of apoptosis genes in autoimmune patients, it has been used in several other clinical settings [34, 35]. Seven target apoptosis gene families and pathways consisting of 93 genes in total were screened (see Table II). cDNA was reverse transcribed from 500 ng of total RNA from seven SLE, five RA patients and seven controls, using the TaqMan Reverse Transcription kit (Applied Biosystems) in a total volume of 50 µl. Reaction mixtures for each sample contained 200 µl TaqMan Universal PCR master mix, 40 µl cDNA (corresponding to 400 ng RNA) in a total volume of 400 µl. Four times 100 µl of the reaction mix was added to each loading port on the array; two samples were analysed per plate. Amplification conditions were 2 min at 50°C to activate uracil-DNA glycosylase (UNG), 10 min at 94.5°C (activation), 40 cycles of denaturation at 97°C for 30 s and annealing and extension at 59.7°C for 1 min. Each sample was measured in duplicate on the same TLDA run. Data were analysed using relative quantification software (SDS2.2-Applied Biosystems) by the threshold cycle (Ct) $\Delta\Delta\text{CT}$ /comparative CT method, and samples were quantified relative to three housekeeping

genes *18S*, *ACTB* (β -actin), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Quality control was achieved by comparison of the duplicate crossing points for each gene per sample.

Statistical Analysis

Differences in pyknosis and apoptosis observed between SLE, RA and healthy controls were analysed by the non-parametric Mann–Whitney *U* test for independent samples. The statistical significance of differential TLDA gene expression among normal, RA and SLE subjects was determined by non-parametric Mann–Whitney *U* test by pair-wise comparison of individual Δct measurements (RA versus controls and SLE versus controls) for each group of samples. The threshold for significance was set at 0.05 (two-tailed) for all tests.

Results

Differences in Apoptotic Activity in Lymphocytes from SLE and RA Patients

The percentage of freshly isolated lymphocytes from each group showing evidence of apoptosis at 0 h was less than 10% (Fig. 1). However, the percentage of lymphocytes that were apoptotic at the beginning of culture (0 h) was moderately higher in patients with RA and SLE than in healthy controls. At 0 h, percentage (the mean \pm 1 SD) of pyknotic lymphocytes (“slide-death”) in healthy controls was 1.7 \pm 1.3% compared with patients with RA (3.9 \pm 2.9%; p <0.05) and SLE (3.6 \pm 2.1%; p <0.05 by Mann–Whitney *U* test).

The percentage of pyknotic lymphocytes after 24 h was significantly greater when compared with 0 h within each

Fig. 1 Slide deaths of lymphocytes. Immediately after lymphocyte isolation (0 h) and after 24 h in culture, cells were stained with Mayer's hemalum, and the percentage of pyknotic cells was determined. Vertical scatter plots depict mean \pm upper and lower 95% CI of the mean (as described in “Methods”)

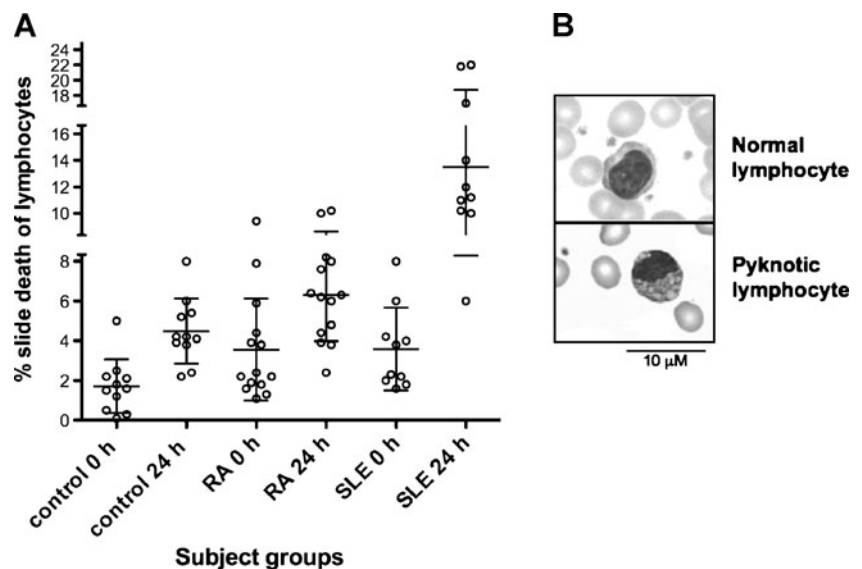


Table II Target Genes and Apoptotic Pathways Screened in SLE, Rheumatoid Arthritis and Healthy Individual Lymphocytes

Apoptotic genes pathway/ families	Gene	Function	Apoptotic genes pathway/ families	Gene	Function
BCL-2 family-up-regulated pathway	<i>BCL2L13, BAK1, BCL2L11, BCL3, BIK, BBC3, BNIP3L, BOK, BCL2L14, BCAP31, BID, HRK, BAD, BAX, BCL10, BCL2L1, MCL1, BNIP3</i>	Promote or induce apoptosis via various mediators (e.g. NF-κB, caspases or p53) Pro- and anti-apoptotic	Caspase family	<i>CASP1, CASP2, CASP 3, CASP4, CASP5, CASP 6, CASP 7, CASP 8, CASP 9, CASP10, CASP14, ESRRL1</i>	Inducers of apoptosis
Death-receptor-regulated pathway	<i>BCL2L2, BCL2L10, Bcl-2, BCL2A1, HIP1, DEDD2, CRADD, DEDD, PEAI5</i>	Promote or induce apoptosis Inhibits CASP8 apoptosis	NF-κB signalling pathway	<i>TBK1, NF-κBIZ, REL, NFKBIA, RELA, NFKB1, NFKB2, NFKBIB, TA-NFKBH, NFKBIE, RELB, HTRA2, DIABLO, BIRC1, BIRC2, BIRC3, BIRC4, BIRC5, BIRC6, BIRC7, BIRC8</i>	Interacts with CASP8 NF-κB activators
p53 activation	<i>PMAIP1 (NOXA)</i>	Induces p53-dependent apoptosis	IAP family	<i>CARD6, CARD 9, CARD15</i>	Apoptotic inhibitors NF-κB activators
Fas signalling pathway (CD95)	<i>FAS (TNFRSF6), FASLG, FADD, CFLAR</i>	Promotes apoptosis Binds to FAS Binds to FAS promoting apoptosis Inhibitor of TNFRSF6-mediated apoptosis	CARD family	<i>NLRP1 (CARD7), CARD4 (NOD1)</i>	Enhances CASP9 apoptosis
TNF receptor pathway	<i>CASP8AP2, LRDD, TNFRSF1A, TNFRSF1B, LTB, TNF, TRADD, TNFSF10, TNFRSF10A, TNFRS10B, TNFRSF21, TNFRSF25, LTA</i>	Activator of CASP8 in FAS-mediated apoptosis Death domain protein interacts with FAS Promote or induce apoptosis Induces inflammation	Kinases	<i>PYCARD, APAF1 (CED4), ICEBERG (CARD18), CHUK, IKKB, RIPK2, IKKε, IKKγ, RIPK1, DAPK1</i>	Pro-apoptotic Caspase 1 inhibitor NF-κB activator Cell death promoter

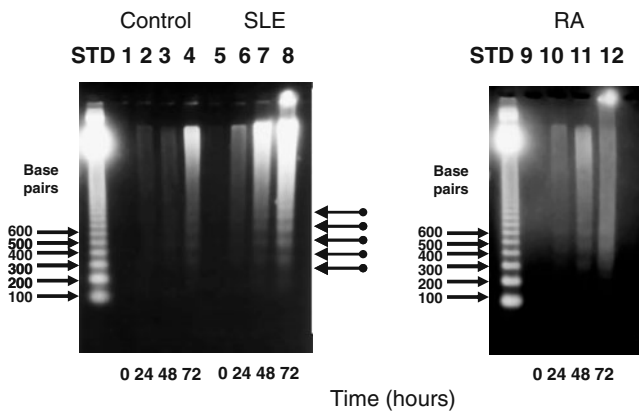


Fig. 2 Gel electrophoresis of DNA. Intranucleosomal DNA samples from all groups were obtained as described in “Methods” and run on a 1.5% agarose gel using a 100-bp ladder as a molecular weight marker (denoted—STD). Samples from each group were isolated either immediately after cell isolation (0 h) or after 0, 24, 48 and 72 h in culture. Lanes 1–4, healthy control; lanes 5–8, SLE patient; and lanes 9–12, RA patient, each 0, 24, 48 and 72 h, respectively. Samples from control and RA samples over 48 h (lanes 1–3 and 9–11) show relatively little DNA fragmentation, but lanes 6, 7 and 8 from the SLE samples show the formation of “ladders” of intranucleosomal DNA fragments at ~150–200-bp intervals (arrowheads). DNA fragmentation could be seen in control samples after 72 h (lane 4) and after 48 h in RA samples (lane 11)

group, as expected (for healthy controls $p < 0.01$, for RA patients $p < 0.01$ and for SLE patients $p < 0.0001$ by Mann–Whitney U test). Patients with SLE showed significant increases in the percentage of pyknotic lymphocytes at 24 h ($13.5 \pm 5.2\%$) when compared with lymphocytes from healthy controls at 24 h ($4.7 \pm 1.6\%$; $p < 0.0001$), although percentage pyknosis in patients with RA at 24 h ($6.3 \pm 2.2\%$) was not significantly greater than controls at 24 h (see Fig. 1). Agarose electrophoresis of DNA isolated from both healthy and diseased lymphocytes showed the presence of a characteristic DNA “ladder”, reflecting the intranucleosomal fragmentation of DNA associated with apoptosis [24]. Typical examples of DNA fragmentation are shown in Fig. 2. The multiple bands of the ladder became progressively more intense as the culture period increased.

QRT-PCR Analysis of Apoptotic Genes in RA and SLE Patients and Control Lymphocytes

Measurement of apoptotic gene expression in individual RA or SLE patients and age-matched controls was performed by QRT-PCR on 93 differentially expressed genes by TLDA. The results were consistent in that expression of apoptotic genes was more pronounced in lymphocytes obtained from individual SLE patients as compared with healthy controls. As shown in Fig. 3a, QRT-PCR revealed a significant increase in mRNA expression of 11 of the 93 genes screened. Comparing SLE patients with

controls, *BIRC2*, *RELB*, *HTRA2*, *BCL2L14*, death effector domain-containing DNA-binding protein (*DEDD2*), *BAD*, *IKBKB*, *TNFRSF10B*, *BBC3*, *IKBKE* and *BID* were significantly up-regulated, and only one of 93 genes was down-regulated, namely apoptosis-associated speck-like protein containing a CARD (*PYCARD*) in six out of seven patients (Fig. 4). In contrast, only two of 93 genes were up-regulated significantly in RA patients, namely *TNFRSF21* (a member of the large TNF receptors superfamily that binds to TNF ligands) and *DEDD2*, the death effector domain-containing DNA-binding protein (Fig. 3b). Interestingly, (*DEDD2*) mRNA levels were present in control lymphocytes but significantly increased at the mRNA level from all seven SLE patients and five RA patients (by four- and five-fold, respectively). *TNFRSF21* was significantly up-regulated in all RA patients tested compared with controls. No significant increases or decreases were seen in the other 81 apoptotic genes screened (see Table II for genes included in study).

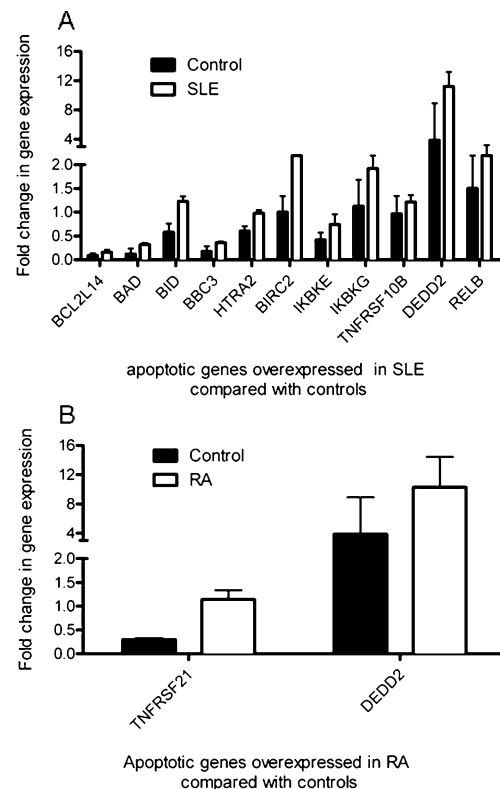


Fig. 3 Genes differentially overexpressed in SLE and RA patients compared with healthy controls. Genes found to show statistically significant differences ($p < 0.05$) in gene expression levels in seven individual SLE patients (a) or and five RA patients (b) compared with seven control subjects are shown. The gene identity is given on the X-axis and the expression of each test gene (fold-change) relative to three house-keeping genes of each gene on the Y-axis. Levels of gene expression in pooled controls are shown in black and those in SLE or RA patients in white. Data are compiled from median fold increase and 75% interquartile ranges of individual Δct values from each individual subject analysed

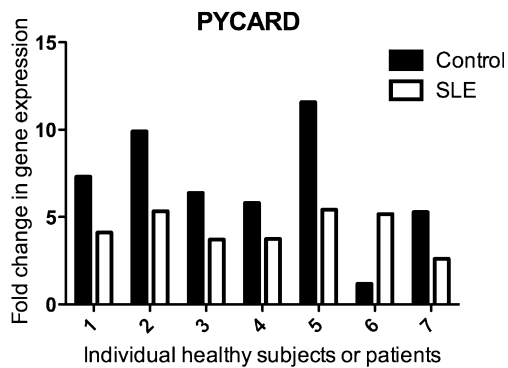


Fig. 4 PYCARD is differentially down-regulated in individual SLE patients compared with healthy controls. PYCARD mRNA expression found to be differentially expressed in seven individual control subjects and in seven individual SLE patients is shown on the graph. The gene identity is given on the X-axis and the relative expression level of each gene on the Y-axis. Levels of gene expression are relative to the average level of three endogenous control genes

Discussion

Our data confirm that freshly isolated lymphocytes show low levels of apoptosis immediately after isolation, and undergo further apoptosis in culture [36]. Immediately after isolation, the extent of pyknosis was significantly raised in both SLE and RA lymphocytes compared with healthy control cells. After 24 h in culture, the difference between SLE and control cells was accentuated, whilst there was no longer a statistically significant difference between RA and control cells, suggesting that lymphocytes from SLE patients may be more susceptible to apoptosis. This supports our previous work in which apoptotic leukocytes (measured by Annexin V binding and nuclear condensation) accumulated in the blood of SLE patients [37].

A few earlier studies have measured apoptosis in peripheral blood lymphocytes from patients with autoimmune diseases by direct morphological observation [24, 38] and demonstrated increased rates of in vitro apoptosis in cultured SLE lymphocytes but no difference of RA lymphocytes when compared with controls. However, the reliability of these methods is uncertain. Flow cytometry relies on the detection of very small percentage differences between cells, whilst the morphologic analysis [38] was restricted to small numbers of counted cells. We therefore suggest that for both methods, analysis of a greater number of lymphocytes is needed, particularly in RA where the majority of activated T cells migrate to the joints, as opposed to the vessels in SLE.

In this study, we wished to quantify fold changes in apoptosis gene expression observed in individual autoimmune patients (prescribed the two most common treatments for SLE (prednisolone) and RA (methotrexate) in our hospitals). In this study, we screened a relatively small number of patients, and one might expect to see random

variation in expression of four to five genes (with a p value set at 0.05) when screening 93 genes. However, we observed the same genes being over expressed in each patient of the patient groupings, which cannot be explained by chance.

A recurrent difference was the overexpression of *DEDD2* in all the RA and SLE patients tested (Fig. 3a, b) irrespective of the different medications in all patients examined compared with healthy controls. *DEDD2* is highly expressed in peripheral blood leukocytes. *DEDD2* has been implicated in a diverse array of diseases including autoimmune lymphoproliferative disease, arthritis and Parkinson disease [39, 40]. The protein is of particular importance in T lymphocytes where it is involved in cell proliferation and activation. *DEDD2* can activate apoptosis by direct interaction of caspases in various cellular locations and may mediate apoptosis induced by activation of cell surface death receptors. For example, *DEDD2* is targeted to the nucleolus of cells by its nuclear localisation signal where *DEDD2* can directly activate caspase 6. *DEDD2* can also bind to caspases 8 and 10 and activate caspase 3 in the cytosol [40]. Caspase 8 translocates possibly via *DEDD2* interaction to the nucleus during Fas (CD95) induced apoptosis. In RA, Fas/FasL-mediated apoptosis of T cells can be inhibited by a number of factors [41], which may explain the lower degree of cell death in cells isolated from RA lymphocytes compared with SLE patients. Moreover, death effector domain proteins may under certain conditions actually prevent lymphocyte cell death [42]. Although the protein induces only moderate apoptosis directly, it can substantially sensitise cells to undergo apoptosis by Fas (CD95/APO-1), Fas-associated death domain (*FADD*) or tumour necrosis-related apoptosis-inducing ligand (*TRAIL*) [43]. Here, we observed greater spontaneous apoptosis in the lymphocytes of SLE patients compared with RA patients despite *DEDD2* being up-regulated in both patient groups. However, it is known that there are increased concentrations of soluble Fas/CD95-ligand present in RA patients that can act to prevent Fas/CD95-induced apoptosis [44]. Interestingly, we observed a five-fold up-regulation of *TRAIL* detected by cDNA array analysis in SLE pooled lymphocytes (data not shown).

Anti-TNF biological agents are highly effective in the treatment of RA [45], but some patients do not respond [46], and the use of anti-TNF therapy can exacerbate autoimmune disease in others [47]. Therefore, a greater understanding of TNF and TNF-related ligands, and their interaction with the 30 or so members of the TNFR superfamily is required. A single member of the TNFR family, *TNFRSF21*, was over expressed in all the RA patients in the present study, none of whom were receiving anti-TNF therapy. *TNFRSF21* is particularly abundant in lymphoid tissue and is capable of inducing apoptosis and engaging in the NF- κ B and JNK pathways [48].

TNFRSF21 utilises the adaptor protein TNFR-associated death domain (*TRADD*) to transmit the death signal. However, we observed no significant up-regulation of *TRADD* mRNA expression in our RA patients compared with controls lymphocytes.

The differential down-regulation of *PYCARD* in SLE patients compared with RA and controls was noteworthy. Autoantibodies to dsDNA are prevalent in SLE. The generation of anti-DNA antibodies may be due to oxidative or other damage to DNA during inflammation [49]. Cells have a distinct system to recognise aberrant DNA inside the cell; toll-like receptors (TLRs), especially TLR9 are adept at binding to aberrant DNA [50]. Several other factors modulate TLR9-mediated signalling, including *PYCARD*. Therefore, the production of *PYCARD* appears to play a crucial role in the recognition of DNA, acting as an adaptor molecule for activation of caspase 1. Defective recognition of self-DNA by TLR9 and other factors associated with the inflammasome, such as *PYCARD*, may contribute to that part of the pathogenesis of SLE associated with DNA damage. This is the first observation of reduced *PYCARD* mRNA levels in SLE, and given the recent findings that deficiencies in *PYCARD* are critical in T cell survival and the development of experimental autoimmune diseases, terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labelling warrants further investigation [51]. The recent work of Habib et al. [52] demonstrated that T lymphocytes in patients with SLE are more prone to apoptosis than T lymphocytes from healthy controls or RA patients in the presence of TNF- α via differences in the TNF- α signalling pathway. It is therefore of interest in this study that SLE lymphocytes are more susceptible to apoptosis, and a member of this signalling pathway *TNFRSF10B* is significantly up-regulated in SLE lymphocytes and not RA patients or healthy controls.

The quantification of the differences between individual patients by QRT-PCR pinpointed altered mRNA expression corresponding to a number of genes, which may help explain the differences in apoptosis observed in different disease states. Perhaps surprising was the select few apoptotic genes that were either up- or down-regulated observed in SLE and RA patients compared with healthy controls. There is no doubt that the therapeutic medications each patient was taking prior to cell isolation may influence the overall results. However, irrespective of treatment, all individual SLE and RA patients presented with up- or down-regulation of the same specific genes, indicating that their drug treatment alone (which differed for individual patients) was not the sole influence for differences in apoptotic gene expression at the mRNA level.

Finally, in this current study, the *in vitro* increase in pyknotic lymphocytes isolated from SLE patients at 24 h is very marked compared with lymphocytes isolated from

healthy subjects and RA patients under identical culture conditions. This suggests that SLE lymphocytes may undergo more rapid apoptosis by intrinsic regulatory pathways. If this greater rate of apoptosis occurs *in vivo*, even if cells are efficiently removed at the same rate as in healthy subjects, the fact that more cells are undergoing apoptosis would lead to a greater accumulation of such cells in the circulation independently of specific defects in the ability to recognise and remove apoptotic cells in individual patients. The identification and up-regulation of a specific and limited number of apoptotic regulatory genes, particularly in the lymphocytes of SLE patients, would add support to this possibility. Further work is required to determine if the genes described here contribute to resistance or increased susceptibility of lymphocytes to apoptosis in these divergent diseases.

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