Increased Rate of Apoptosis and Decreased Expression of bcl-2 Protein in Peripheral Blood Lymphocytes from Patients with Active Systemic Lupus Erythematosus

Eric YT Chan, Stanley CW Ko and Chak S Lau

Apoptosis (programmed cell death) is a form of cell death characterized by cell shrinkage, nuclear condensation, and surface blebbing not accompanied by inflammatory cell infiltration. This is in contrast to necrosis in which cell lysis and digestion of cellular contents are early events. Apoptosis is initiated by ligand-receptor interactions that are highly regulated. Proto-oncogenes and tumour suppressor genes, including bcl-2 which inhibits apoptosis, myc and p53 which enhance apoptosis, operate at different points along the path to apoptosis. Recently, there has been a lot of interest in studying the role of apoptosis in the pathogenesis of autoimmune diseases. In the MRL-lpr/lpr mouse, a structural abnormality in the fas gene resulting in decreased expression of the fas protein was linked to decreased apoptosis of peripheral lymphocytes and gradual accumulation of these lymphocytes. This was thought to increase the lifespan of autoreactive cells resulting in autoimmune disease manifestations. In the present study, we sought to analyse peripheral blood lymphocytes from SLE patients with various levels of disease activity and investigate 1) the cytoplasmic expression of the bcl-2 protein and 2) the in vitro apoptotic rate by placing lymphocytes in culture for 48 hours without stimulation. A decreased expression of bcl-2 and an increased apoptosis were found in patients with active lupus.

SUMMARY Defective regulation of apoptosis may play a role in the development of autoimmune diseases, and the proto-oncogene bcl-2 is known to inhibit cells from undergoing apoptosis. We studied the rate of apoptosis and the expression of bcl-2 in peripheral blood lymphocytes of patients with systemic lupus erythematosus (SLE). A lower proportion of lymphocytes were bcl-2+ in SLE patients with active disease (median 84.9%) than in patients with inactive disease or normal (medians 95.3% and 97.1% respectively, p<0.05). The rate of apoptosis of freshly isolated PBL was significantly higher in SLE patients than in normal (medians 1.2% vs 0.5%, p<0.05). After 48-hour culture the apoptotic rate was further increased in SLE patients, particularly those with active disease (SLE overall 34.2%, active 62%, inactive 27.5%, normal 11.5%). These findings support the theory that in SLE patients increased apoptosis may provide a source of extracellular nuclear antigens which stimulate the autoimmune response and form immune complexes with autoantibodies.

PATIENTS AND METHODS

Patients

Patients who met 4 or more criteria of the American College of Rheumatology classification of SLE were recruited from the Department of Pathology and Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong. Correspondence: Eric Chan.
partment of Medicine, University of Hong Kong. All patients with inactive disease were receiving a low dose (5-10 mg/day) of prednisone during the time of study. Some were also taking 50-100 mg/day azathioprine. Those who had active disease were studied prior to the augmentation of their steroid dosage; hence there was no difference in drug treatment from those with inactive disease. For comparison, healthy volunteers of similar age and sex distribution were included.

Disease activity

The Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)\(^\text{10}\) was used to assess the disease activity of each patient during the study. Clinical parameters were scored by 2 of the authors who followed the progress of all patients. Laboratory tests were performed by the Clinical Pathology laboratories in the same hospital. Anti-dsDNA antibodies were assayed by ELISA using the International Reference Standard for anti-dsDNA antibody and C3, C4 were measured by nephelometry.

Flow cytometry staining and analysis

Cytoplasmic expression of bcl-2 were studied by standard indirect flow cytometry technique. Briefly, peripheral blood mononuclear cells were obtained by Ficoll-hypaque sedimentation. The cell membrane was permeabilised by incubation with saponin (0.25% in phosphate-buffered saline for 30 minutes). The cells were then treated with a mouse IgG monoclonal antibody against bcl-2 (Dako A/S, Denmark) the binding of which was detected after washing by a fluorescein-labelled anti-mouse IgG antibody (Silenus, Australia). For isotypic control, a mouse monoclonal antibody of the same isotype but not reacting with human lymphocytes was used. After staining, the cells were analysed by a Coulter Profile II flow cytometer. Only cells with forward and side scatter characteristics of lymphocytes were gated. The cut-off level was set to include 2% of the isotypic control cells. The percentage of positive cells above this cut-off level and the mean fluorescence channel number were recorded.

Flow cytometry quality control procedure

Optical alignment of the flow cytometer was set daily using the standard beads DNA check (Coulter, Hialeah, FL 33014, USA). The machine settings were counter-checked before each assay by the standard beads CaliBRITE (Becton Dickinson, San Jose, CA 95131, USA) which gave a mean fluorescence level of 23.5 (standard deviation 0.45). Frozen mononuclear cells were stained in parallel with test samples during each assay.

Cell culture and staining of apoptotic cells

Peripheral blood mononuclear cells were isolated by Ficoll-hypaque sedimentation and monocytes were removed by plastic adherence. Lymphocytes were cultured in RPMI (Gibco) supplemented with 10% foetal calf serum for 48 hours after which cytosmears were prepared. An in situ apoptosis detection kit (Oncor, 209 Perry Parkway, Gaithersburg, MD 20877, USA) was used to label cells with DNA fragmentation as a result of apoptosis. In brief, the smear was incubated with residues of digoxigenin-nucleotide which were added to DNA by terminal deoxynucleotidyl transferase (TdT). Anti-digoxigenin antibody carrying a fluorescein label was then added and the number of fluorescent cells was counted.

Statistics

Two sample comparisons were performed by the Wilcoxon technique. Correlation between 2 samples was studied by the Spearman rank correlation test. Both are non-parametric tests.

RESULTS

Expression of bcl-2 in cytoplasm of PBL

Thirty SLE patients and 20 normal individuals were recruited in this study and results are shown in Table 1. A significantly lower percentage of PBL were bcl-2\(^+\) in SLE patients than in normal (medians of 90.6% vs 97.1%, \(p < 0.05\)). The difference was more marked between those with active SLE (SLEDAI >9) and normal (medians of 90.6% vs 97.1%, \(p < 0.05\)). There was no significant difference between those with inactive SLE (SLEDAI <5) and normal. The difference in antigenic level (reflected by mean fluorescence level) of bcl-2\(^+\) cells between SLE patients and normal was not statistically significant.

Apoptosis of PBL in culture

The rate of apoptosis after 2-day culture without stimulation were studied in 21 SLE patients and
11 normal individuals. A small number of apoptotic cells (<2%) were seen on Giemsa-stained cytosmears prepared from both normal and patient cells. A more sensitive commerical fluorescence kit which labels cells with DNA fragmentation was therefore used to detect apoptotic cells on cytosmears. These results together with total lymphocyte counts are shown in Table 2. On day 0, the percentage of apoptotic PBL was significantly higher in SLE patients than in normal (medians 1.2% vs 0.5%, p < 0.01). The difference was greater between active SLE patients (median 2%) and normal (p < 0.001). After 2-day culture, the percentage of apoptotic cells from active SLE patients rose to a median of 65% and this was significantly greater than that of inactive SLE patients (median 27.5%, p < 0.01). The latter was in turn greater than normal control (median 11.5%, p < 0.01).

Neither the apoptotic rate on day 0 nor that on day 2 correlated with the absolute lymphocyte count. However, the total lymphocyte count was significantly higher in in-

**DISCUSSION**

We have reported here an increased apoptosis and a decreased bcl-2 expression in PBL from patients with SLE than normal. Our work also confirms data of others that normal lymphocytes underwent apoptosis when placed in tissue culture. The difference in apoptosis between SLE patients and normal was found with freshly isolated lymphocytes but was much greater with lymphocytes in tissue culture for 48 hours. An increased rate of apoptosis was similarly reported in autoimmune (MRL-lpr/lpr) mice and in SLE patients. In the latter study, apoptosis was shown not due to corticosteroid, cytotoxic drugs or the isolation procedure and the increase in apoptotic rate correlated with SLE disease activity. In our study, as there was no difference in drug treatment between active and inactive patients but a significant difference in apoptosis, corticosteroid or cytotoxic drugs were unlikely to be responsible for the in-

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**Table 1.** Medians (lower and upper quartiles) of bcl-2 expression in cytoplasm of PBL.

<table>
<thead>
<tr>
<th>Sample group</th>
<th>% bcl-2 in PBL</th>
<th>Mean fluorescence level of bcl-2 in PBL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>97.1 (93.7-98.6)</td>
<td>49.0 (41.4-55.7)</td>
</tr>
<tr>
<td>SLE overall</td>
<td>90.6 (79.3-97.1)</td>
<td>48.3 (42.2-56.2)</td>
</tr>
<tr>
<td>Inactive SLE (SLEDAI &lt; 5)</td>
<td>95.3 (83.4-98.7)</td>
<td>55.2 (48.3-60.6)</td>
</tr>
<tr>
<td>Active SLE (SLEDAI &gt; 9)</td>
<td>84.9 (79.3-90.6)</td>
<td>37.7 (34.7-53.6)</td>
</tr>
</tbody>
</table>

**Table 2.** Medians (lower and upper quartiles) of percentages of apoptotic PBL after 2-day culture

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Lymphocyte count (x10⁹/l)</th>
<th>% apoptotic PBL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 2</td>
</tr>
<tr>
<td>Normal</td>
<td>Not done</td>
<td>0.5 (0-0.8)</td>
</tr>
<tr>
<td>SLE overall</td>
<td>0.9 (0.67-1.5)</td>
<td>1.2 (0.8-2.5)</td>
</tr>
<tr>
<td>Inactive SLE (SLEDAI &lt; 5)</td>
<td>1.2 (0.8-1.75)</td>
<td>1 (0.5-1.65)</td>
</tr>
<tr>
<td>Active SLE (SLEDAI &gt; 9)</td>
<td>0.62 (0.6-0.8)</td>
<td>2 (1.5-2.5)</td>
</tr>
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</table>
creased apoptosis in active lupus patients. These data, together with the defect in bcl-2 expression found, strongly suggest the increased apoptosis is an intrinsic abnormality of lymphocytes in SLE patients. The aetiology of this intrinsic abnormality is uncertain but it is known that apoptosis is intimately linked to cell stimulation and proliferation and in SLE the immune system is hyper-stimulated. We and several others have found an increase in the activation antigen CD25 (interleukin-2 receptor αchain) on PBL of SLE patients. 

It has been postulated that the apoptotic lymphocytes may provide a source of extracellular nuclear antigens which drive the immune response and to allow the formation of immune complexes. Systemic lupus erythematosus, as a prototypic autoimmune disease, is characterised by an autoantibody response to several intracellular antigens. Evidence exists that production of these autoantibodies is antigen-driven and these antigens must be exposed to the immune system in the extracellular space. Although circulating nucleosomal DNA was found in plasma of some SLE patients the release of these intracellular antigens has long been a puzzle. Casciola-Rosen et al. demonstrated apoptotic blebs induced by ultraviolet light on keratinocytes contained nuclear and cytoplasmic antigens. Release of nucleosomes into the extracellular milieu during apoptosis was also demonstrated in vitro by Emlen et al. These released nucleosomes was shown to stimulate lymphocytes, increasing both DNA and immunoglobulin synthesis and induce IL-6 production. In vivo apoptosis may therefore contribute to increased polyclonal B cell stimulation and autoantibody responses through the release of nuclear autoantigens.

Our finding of decreased bcl-2 protein expression in cytoplasm of blood lymphocytes in active SLE was consistent with the increased rate of apoptosis. Kumagai et al. also reported a decrease in bcl-2 expression in active SLE patients but in the reports of Aringer et al. and Oshako et al. peripheral T cells were studied and increased bcl-2 expression was found. It might be that bcl-2 is differentially expressed in various lymphocyte subsets and/or the dysregulation is different in various disease subgroups. An increased bcl-2 level supports the attractive theory that autoreactive cells in SLE patients are not peripherally deleted and have increased survival. Transgenic mice which over-expressed bcl-2 in B cells showed polyclonal B cell expansion and developed an autoimmune disease resembling SLE a few months after birth. On the other hand, increased apoptosis and decreased bcl-2 expression of non-autoreactive lymphocytes may be a source of nuclear autoantigens as discussed above. These two theories are not mutually exclusive and both may contribute to the pathogenesis of SLE.

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REFERENCES


