ABSTRACT

The bcl-2 oncogene is expressed in lymphoid and myeloid cells as well as in neurons and several types of epithelial cells and inhibits programmed cell death (apoptosis). Deregerulation of the bcl-2 gene (1, 2) reflects recombination of the oncogene on chromosome 18 with the immunoglobulin heavy chain gene locus on chromosome 14 (3), resulting in deregulation of expression inappropriately high levels of bcl-2 mRNA and protein (14-16). bcl-2 appears not to act directly as a growth promoting factor, but there is ample evidence that it can protect cells against apoptosis (programmed cell death) by arresting cells in the G0/G1 phase of the cell cycle. This follows from experiments in vitro, in which protection from cell death by growth factor deprivation in factor-dependent cell lines was prevented (7-11). Similar antipotopotic effects were demonstrated for bcl-2 expressing thymocytes treated with a variety of cytotoxic agents (12-15). Transgenic mice bearing a Eph-bcl-2 minigene develop a massive polyclonal population of small resting B-lymphocytes (14-16). These recirculating B-cells apparently accumulate due to an extended life span rather than because of increased proliferation. Only after a long latency period, some of the animals develop a lymphoma of high grade malignancy (17, 18), a strong indication that bcl-2 activation is not sufficient for complete malignant transformation of lymphoid cells. In the physiological, nonmalignant situation, bcl-2 may play an important role in the maintenance of B-cell memory (16, 19, 20).

MATERIALS AND METHODS

Materials. Human peripheral blood lymphocytes from normal volunteers were isolated from whole blood by Ficoll density gradient centrifugation. Fresh and cryopreserved samples of B chronic lymphocytic leukemia cells, with high bcl-2 expression, as assayed by immunohistochemistry on frozen sections and cytocentrifuge preparations, were analyzed. The DoHH2 cell line (29), a t(14;18) bearing cell line with high bcl-2 expression, as also indicated by immunohistochemistry, was cultured for 1-3 days before further processing.

Preembedding Immunohistochemistry. Cell suspensions were fixed for 1 h in 2% PLP at 4°C and the fixed cells were pelleted in 10% gelatin in PBS. The pellet was postfixed in PLP overnight. Gelatin blocks were snap frozen in isopentane; 7-μm frozen tissue sections were made and incubated with monoclonal antibodies (anti-bcl-2.124, 1:5 and anti-CD45, 1:5) overnight, washed in phosphate-buffered saline; DAB, diaminobenzidine; CLL, chronic lymphocytic leukemia.

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RESULTS

The antibody anti-bcl-2α.124 was used in a preembedding technique on ultrathin cryostat sections and cytospin preparations and was visualized by DAB complexes. The preparations were analyzed by reflection contrast light microscopy. Normal peripheral blood lymphocytes, which are known to express bcl-2 protein at moderate levels, showed intracytoplasmic bcl-2 immunoreactivity (Fig. 1a). Electronmicroscopy of the same material showed a patchy distribution of DAB complexes at the circumference of the mitochondria (Fig. 1a, b and c). The cristae and matrix did not show bcl-2 immunoreactivity. bcl-2 could also be demonstrated on the nuclear envelope and along the cell membrane (Fig. 1c). In some sections, the distribution along the nucleus was patchy and seemed to be associated with nuclear pores. The patchy distribution of DAB label was emphasized by digitalized image analysis (Fig. 1d).

In CLL cells, which have a much stronger bcl-2 expression than normal blood lymphocytes, a similar staining pattern could be found with the anti-bcl-2α.124 antibody, except for additional patchy staining of the endoplasmic reticulum, that was not obviously membrane related, and of occasional positive small vesicles. This could reflect the (enhanced) production and processing of bcl-2 protein in these cells. Both fresh CLL cells and CLL cells which had been cryopreserved in dimethyl sulfoxide (which may alter membrane structures and therefore may influence the distribution of proteins within these membranes) showed a similar staining pattern.

In the t(14;18) carrying cells of the DoHH2 cell line (29) bcl-2 immunoreactivity was found in a similar pattern as in peripheral blood lymphocytes, with patchy staining of the mitochondrial circumference and staining of the nuclear envelope and the plasma membrane.

To exclude the possibility that a limited access of antibody and DAB molecules caused the staining of mitochondrial outer structures and the lack of staining of the mitochondrial inner structures, ultrathin cryosections were also analyzed in a postembedding technique using the same anti-bcl-2α.124 antibody and immunogold labeling. This showed a similar distribution of label (Fig. 2, a and b).

Relative staining of organelles and membranes was quantitated in 32 lymphocytes (17 and 15 cells in two independent experiments; Table 1) and as internal controls for background staining, in 5 granulocytes, since no bcl-2 expression has been reported in granulocytes (21, 35). Both experiments confirmed the preferential staining of the
Fig. 2. a, postembedding immunogold/bcl-2-staining with antibody anti-bcl-2a.124 of blood peripheral lymphocytes. bcl-2 distribution as visualized by 10-nm immunogold labeling shows essentially the same distribution as seen with the DAB technique. Preferential staining along the mitochondrial outer circumference is seen. × 22,000. b, immunoelectron microscopy as in Fig. 2a staining of the nuclear envelope; an association with nuclear pores is suggested. × 36,000. c, postembedding immunogold/bcl-2-staining with antibody 4D7 of blood peripheral lymphocytes shows the same distribution of label along the mitochondrial circumference. × 26,000. d, immunoelectron microscopy as in Fig. 2a showing association with the nuclear envelope. Magnification × 36,000. (A retraction artefact is present along a membrane.) e, postembedding immunogold/bcl-2-staining with antibody anti-bcl-2a.124 of blood peripheral lymphocytes, visualized with 15-nm colloidal gold. Distribution along mitochondrial and nuclear membranes is seen. × 66,000. f, postembedding immunogold/bcl-2-staining with antibody anti-bcl-2a.124 of blood peripheral lymphocytes, visualized with 10-nm colloidal gold with a similar distribution as in Fig. 2e (a granular proteinaceous background is seen).
bcl-2 PROTEIN LOCALIZATION BY IMMUNOELECTRON MICROSCOPY

Table 1: Quantitation of immunogold staining in peripheral blood leukocytes.

In 32 lymphocytes and 5 granulocytes in two independent experiments, relative surfaces of the cell membrane, cytoplasm, mitochondria, nuclear membrane, and nucleus were assessed by point-hit counting. This was performed by overlaying electron micrographs of standardized magnification and photographic enlargement with a standardized grid of arbitrary grid size. The (relative) surface areas of the different cellular compartments (nucleus, cytoplasm, mitochondria, membranes) were determined by point-hit analysis, whereby the points of intersection of the overlaying grid were attributed to these cellular compartments and quantitated. Note that the differences in surface areas between the two experiments in Table 1 are due to different photographic enlargements. Due to the use of 3-step immunoassay, some displacement of gold particles away from the exact location of the antigen is to be expected. Gold particles were attributed to membrane structures within this margin of approximately 60 nm. Surface areas were assessed with the same margin by point-hit counting.

<table>
<thead>
<tr>
<th></th>
<th>Surface area (relative surface)</th>
<th>No. of particles</th>
<th>Particles/area</th>
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</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lymphocytes (n = 17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondria</td>
<td>250 (4.6)</td>
<td>113</td>
<td>0.516</td>
</tr>
<tr>
<td>Matrix</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleus</td>
<td>3760 (54.8)</td>
<td>128</td>
<td>0.034</td>
</tr>
<tr>
<td>Nuclear envelope</td>
<td>176 (3.1)</td>
<td>81</td>
<td>0.460</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2280 (33.8)</td>
<td>174</td>
<td>0.076</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>206 (3.6)</td>
<td>39</td>
<td>0.189</td>
</tr>
<tr>
<td>Granulocytes (n = 5)</td>
<td></td>
<td></td>
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<tr>
<td>Nucleus</td>
<td>1750 (44.7)</td>
<td>56</td>
<td>0.032</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2160 (55.3)</td>
<td>39</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
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<tr>
<td>Lymphocytes (n = 15)</td>
<td></td>
<td></td>
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<tr>
<td>Mitochondria</td>
<td>512 (4.2)</td>
<td>196</td>
<td>0.382</td>
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<tr>
<td>Nucleus</td>
<td>6173 (51)</td>
<td>663</td>
<td>0.107</td>
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<tr>
<td>Nuclear envelope</td>
<td>473 (3.9)</td>
<td>206</td>
<td>0.435</td>
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<tr>
<td>Cytoplasm</td>
<td>4382 (36)</td>
<td>295</td>
<td>0.067</td>
</tr>
<tr>
<td>Cell membrane</td>
<td>653 (5.4)</td>
<td>132</td>
<td>0.202</td>
</tr>
</tbody>
</table>

a The data presented in Table 1, Experiment 2, were published as part of the proceedings of the workshop “Mechanisms in β-cell neoplasia (47).”

b Numbers in parentheses, percentage.

Mitochondrial circumference versus the matrix, and staining of the nuclear envelope (Table 1). The diffuse staining of lymphocyte nuclei (0.034 and 0.107 particles/surface area, respectively) could be attributed to background staining, especially since granulocytes showed a similar level of background staining (0.032 particles/surface area in experiment 1). In contrast, the staining of cytoplasm was significantly higher in lymphocytes than in granulocytes (0.076 particles/surface area versus 0.018 particles/surface area in experiment 1). The two experiments differed in some minor technical details, accounting for differences in nuclear background staining.

To underscore the specificity of the staining patterns, additional antibodies were used, raised against a different bcl-2 oligopeptide (amino acids 61–76). The monoclonal antibody 4D7 showed the same immunolocalization pattern for bcl-2 (Fig. 2, c and d). The polyclonal antibody 9718 also showed the same distribution, although at a much lower staining level, rendering quantitation unreliable. Furthermore, negative controls in which the first step had been omitted did not show any signal. Using the preembedding DAB technique, staining with an unrelated antibody (common leukocyte antigen, CD45) showed a completely different distribution of label, i.e., staining of the cell membrane.

**DISCUSSION**

Membrane separation techniques have suggested that bcl-2 protein is associated with mitochondrial membranes (9). Other groups, however, have found that anti-bcl-2 antibodies bind not only to mitochondria, but also to cell and nuclear membrane fractions (19, 23, 25).

Using reflection-contrast light microscopy, we could confirm the patchy intracytoplasmic immunoreactivity in lymphocytes, CLL cells, and cells of a lymphoid line carrying a t(14;18) translocation (DoHH2) (29). Using immunoelectron microscopy, both with pre- and postembedding labeling techniques, we showed immunoreactivity on the circumference of the mitochondria, on the nuclear envelope, and also on the cell membrane. There was no staining of the mitochondrial cristae and matrix. Notably, antibodies raised to different parts of the bcl-2 protein (against amino acids 41–54 and 61–76, both located in the hydrophilic portion of the protein) showed identical results, underscoring the specificity of the method. Also, both monoclonal and polyclonal antibodies were used. There was no essential difference in staining patterns of lymphocytes versus normal peripheral blood lymphocytes on one hand and CLL cells and DoHH2 cells with a known high expression of bcl-2 on the other hand. In CLL, some additional staining of the endoplasmic reticulum and intracytoplasmic vesicles was seen. Using postembedding techniques with quantification of immunogold particles, there was also some increase of cytoplasmic staining in normal blood lymphocytes as compared to granulocytes in the same preparations. All in all, these data are in line with similar studies of Monaghan et al. (36).

bcl-2 immunostaining of the mitochondrial outer structures showed a non-homogeneous, patchy distribution, suggestive of association with contact zones. These are highly stable areas in which the mitochondrial outer and inner membrane are cross-linked by transmembrane proteins and where transportation across the membranes is mediated (37–40). Association of bcl-2 to mitochondrial contact sites might explain the difference between our results and those obtained by Hockenberry et al. (9), in which bcl-2 immunoreactivity was associated with mitochondrial inner membrane fractions. Because of the high stability of mitochondrial contact zones, these structures remain associated with the inner membrane fraction after shearing off from the outer membranes in membrane separation techniques (38). This may give the impression that the proteins within these zones are actually associated with inner membranes.

Interestingly, the distribution of bcl-2 was also patchy on the nuclear membrane, sometimes suggesting an association with nuclear pores, which are also sites of transmembrane transport. It may be that bcl-2 is involved in a homologous way in mitochondria and in the nuclear envelope. This view is emphasized by experiments of Jacobs son et al., who showed overexpression of bcl-2 in the nuclear envelope in cells with a defective mitochondrial function (41). Moreover, the anti-apoptotic effect of bcl-2 was retained in these cells.

bcl-2 may be involved in transmembrane transport in different organelles. The import mechanism of the endoplasmic reticulum, mitochondria as well as the nucleus are built up along similar lines, encompassing signal sequences, signal receptors, and translocation apparatus (42). Signal sequences in mitochondria in yeast and Neurospora crassa have been defined and do not bear similarities to bcl-2 (38, 43, 44). Alternatively, bcl-2 might be implicated in the control of intracellular Ca²⁺ levels since both mitochondria and the nuclear envelope share important functions in the regulation of Ca²⁺. Ca²⁺-mediated activation of DNA-endonucleases have been described for a variety of cytotoxic pathways, including glucocorticoid-induced thymocyte lysis and growth-factor deprivation-induced apoptosis in growth factor-dependent cell lines (45, 46).

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