Subcellular Localization of the bcl-2 Protein in Malignant and Normal Lymphoid Cells

Daphne de Jong,1 Frans A. Prins, David Y. Mason, John C. Reed, Gertjan B. van Ommen, and Philip M. Kluin

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). Deregulation by the (t(14;18)) translocation in lymphoid malignancies induces inappropriate cell survival and serves as one of the steps toward a fully malignant behavior. Using pre- and postembedding immunoelectron microscopy in normal and neoplastic lymphocytes, we demonstrate bcl-2 immunoreactivity to the mitochondrial outer circumference and the nuclear envelope and to a lesser degree to the cell membrane. Mitochondrial staining was patchy, reminiscent of mitochondrial contact zones. Additionally, there was a suggestion of association with nuclear pores. In these regions, transmembrane transport is mediated. This may suggest that bcl-2 exerts its function in this process.

INTRODUCTION

Human follicle center cell lymphomas commonly bear a (t(14;18)) translocation, involving the bcl-2 gene (1, 2). The translocation reflects recombination of the oncogene on chromosome 18 with the immunoglobulin heavy chain locus on chromosome 14 (3), resulting in deregulation of expression and inappropriately high levels of bcl-2 mRNA and protein (4-6). 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 antiapoptotic effects were demonstrated for bcl-2 expressing thymocytes treated with a variety of cytotoxic agents (12-13). Transgenic mice bearing a Eα-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 the B-cell memory (16, 19, 20).

The bcl-2 gene encodes two proteins, bcl-2α and bcl-2β, which are identical except for their carboxy terminus (21, 22, 23). The bcl-2α protein consists of 239 amino acids (M, 26,000) and is most abundantly present in lymphoid cells. Protein structure analysis suggests that the bcl-2α protein has a hydrophobic carboxy-terminal membrane-spanning motif and an extensive hydrophilic stretch of amino acids, which is presumed to protrude into the cytoplasm from the membrane surface (23-25). No signal sequences have been demonstrated. Recently, novel oncogenes involved in the regulation of apoptosis have been identified (Rox, Bcl-x and Mcl-1) (26-28). These are the first identified genes that show considerable sequence homology to bcl-2.

Membrane separation techniques and immunoprecipitations have been used by several groups to study the localization of the protein within the cell. One group detected bcl-2 protein in mitochondrial membranes, while others claimed that bcl-2α protein was associated with the nuclear envelope, plasma membrane, and endoplasmic reticulum of cells with a (t(14;18)) translocation (9, 19, 23, 25).

Here we determine the intracellular localization of the bcl-2 protein both in malignant and non-malignant lymphoid cells by using reflection-contrast light microscopical and immunoelectron microscopic techniques.

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 assessed by immunohistochemistry on frozen sections and cytotoxicity 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 prepared from frozen tissue sections were made and incubated with monoclonal antibodies (anti-bcl-2.124, 1:5 and anti-CD45, 1:5) overnight, washed in PBS-ovalbumin, incubated in peroxidase-conjugated rabbit anti-mouse (1:50, Dakopatts, Copenhagen, Denmark) for 2 h, washed again, and reacted with DAB medium for 15 min. Sections were postfixed in Os04 (reduced), dehydrated, and embedded in EPON 812. Sequential ultrathin section were cut and placed either on glass slides for reflection contrast microscopy or on grids for electronmicroscopy (30). No poststaining was performed. The preparations were examined in a Leitz Orthoplan microscope (reflection-contrast light microscopy) and in a Philips CM-10 electron microscope. A similar procedure was performed on cytosin preparations, fixed in PLP (10 min). A computer-generated gray scale image from two samples was made on a mini-magiscan (IAS 25/125, Joyce-Loebl Ltd., England).

Postembedding Immunohistochemistry. Cells were fixed, embedded in gelatin capsules, and fixed in buffered 4% paraformaldehyde-0.1% glutaraldehyde; 2.3 μm thick/μcm was added/PBS was added to 1-mm3 fragments which were subsequently snap frozen; 60-nm sections were made by using a Reichert-Jung cryoultramicrotome. Sections were placed on grids and immunohistochemistry was performed in situ, using a 3-step immunostaining technique, using rabbit anti-mouse immunoglobulin, followed by goat anti-rabbit immunoglobulin conjugated to 10- and 15-nm colloidal gold (31, 32). The grids were incubated in methylcellulose and uranyl acetate and examined in a Philips CM-10 electron microscope. Quantititation of immuno-gold labeling to subcellular compartments was performed on 37 individual cells in total (15 and 17 lymphocytes in two independent experiments as well as 5 granulocytes in one of these).

Antibodies to bcl-2 used in these assays were: anti-bcl-2a.124 (monoclonal antibody, raised against a synthetic oligopeptide of amino acids 41-54) (33), 9718 (polyclonal antibody raised against amino acids 61-76) (10), and 4D7 (monoclonal antibody raised against amino acids 61-76) (34).

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2 The abbreviations used are: PLP, paraformaldehyde-lysine-periodate; PBS, phosphate-buffered saline; DAB, diaminobenzidine; CLL, chronic lymphocytic leukemia.
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. 1, 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-2.124 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. 2e 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-2.124 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-2.124 of blood peripheral lymphocytes, visualized with 10-nm colloidal gold with a similar distribution as in Fig. 2e (a granular proteinaceous background is seen).
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, whereas 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 immunositivity, some dislocation 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>Experiment</th>
<th>Surface area (relative surface)</th>
<th>No. of particles</th>
<th>Particles/area</th>
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<tr>
<td>Lymphocytes (n = 17)</td>
<td>Mitochondria</td>
<td>Circumference 250 (4.6)</td>
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<td>Matrix</td>
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<tr>
<td></td>
<td>Nucleus</td>
<td>3760 (54.8)</td>
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<tr>
<td></td>
<td>Nuclear envelope</td>
<td>176 (3.1)</td>
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<td></td>
<td>Cytoplasm</td>
<td>2280 (33.8)</td>
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<tr>
<td></td>
<td>Cell membrane</td>
<td>206 (3.6)</td>
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<tr>
<td>Granulocytes (n = 5)</td>
<td>Nucleus</td>
<td>1750 (44.7)</td>
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<tr>
<td></td>
<td>Cytoplasm</td>
<td>2160 (55.3)</td>
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</tbody>
</table>

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 pattern between 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 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, whereas 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 immunositivity, some dislocation 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.

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<th>No. of particles</th>
<th>Particles/area</th>
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<td>Nucleus</td>
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<td>Granulocytes (n = 5)</td>
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<td>1750 (44.7)</td>
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