Prognostic Significance of bcl-2 Protein Expression in Aggressive Non-Hodgkin’s Lymphoma

By Olivier Hermine, Corinne Haioun, Eric Lepage, Marie-Françoise d’Agay, Josette Briere, Caroline Lavignac, Georges Fillet, Gilles Salles, Jean-Pierre Marolleau, Jacques Diebold, Felix Reyes, Philippe Gaulard, for the Groupe d’Etude des Lymphomes de l’Adulte (GELA)

Little is known about the expression of bcl-2 protein in intermediate and high grade non-Hodgkin’s lymphoma (NHL) and its clinical and prognostic significance. We performed immunohistochemical analysis of bcl-2 expression in tumoral tissue sections of 348 patients with high or intermediate grade NHL. These patients were uniformly treated with Adriamycin, cyclophosphamide, vindesine, bleomycin, and prednisone (ACVBP) in the induction phase of the LNH87 protocol. Fifty eight cases were excluded due to inadequate staining. Of the 290 remaining patients, 131 (45%) disclosed homogeneous positivity (high bcl-2 expression) in virtually all tumor cells, whereas 65 (23%) were negative and 94 (32%) exhibited intermediate staining. High bcl-2 expression was more frequent in B-cell NHL (105 of 214, 51%) than in T-cell NHL (6 of 35, 17%) (P = .0004), and was heterogeneously distributed among the different histological subtypes. Further analysis was performed on the 191 patients with diffuse large B-cell lymphoma (centroblastic and immunoblastic) to assess the clinical significance and potential prognostic value of bcl-2 expression in the most frequent and homogeneous immunohistological subgroup. High bcl-2 expression, found in 44% of these patients (67 of 151), was more frequently associated with III-IV stage disease (P = .002). Reduced disease-free survival (DFS) (P < .01) and overall survival (P < .05) were demonstrated in the patients with high bcl-2 expression. Indeed, the 3-year estimates of DFS and overall survival were 60% and 61%, respectively (high bcl-2 expression) versus 82% and 78%, respectively (negative/intermediate bcl-2 expression). A multivariate regression analysis confirmed the independent effect of bcl-2 protein expression on DFS. Thus bcl-2 protein expression, as demonstrated in routinely paraffin-embedded tissue, appears to be predictive of poor DFS, in agreement with the role of bcl-2 in chemotherapy-induced apoptosis. It might be considered as a new independent biologic prognostic parameter, which, especially in diffuse large B-cell NHL, could aid in the identification of patient risk groups.

© 1996 by The American Society of Hematology.

The BCL-2 gene was initially discovered by virtue of its involvement in the (t(14;18)(q32;q21) translocation. This chromosomal abnormality, resulting in production of high levels of bcl-2 protein, is observed in the majority of follicular non-Hodgkin’s lymphomas (NHLs) and in about 20% of diffuse large B-cell lymphomas. However, the expression of bcl-2 protein is not restricted to B-cell lymphomas bearing the t(14;18) translocation. Indeed, immunohistochemical studies have shown that, beside follicular lymphomas, a broad spectrum of lymphoid malignancies including chronic lymphocytic leukemia, plasma cell dyscrasia, diffuse large B- and T-cell lymphomas, as well as non-lymphoid tumors of lung, breast, prostate or liver origin, also express the bcl-2 protein. Finally, the bcl-2 protein is also detected in a number of normal tissues, including B lymphocytes of the mantle zone and normal T cells.

Bcl-2 can be regarded as a member of a new category of oncogenes that is involved in cell survival, by blocking programmed cell death, also called apoptosis. Deregulated bcl-2 extends the survival of some interleukin-dependent hematopoietic cell lines when deprived of growth factors. In vivo, bcl-2-Ig transgenic mice express constitutively a high level of bcl-2 protein, and thus accumulate an excess of resting IgM/IgD B cells because of extended survival. Thus, constitutive bcl-2 expression might cooperate with the activation of other oncogenes, such as c-myc, in a multistep development of lymphomas.

It has been shown that apoptosis occurs in a majority of lymphoid cells when treated by various antineoplastic agents commonly used in the treatment of NHLs. Recent in vitro studies on murine and human leukemia cell lines have demonstrated that, although not preventing suppression of cell proliferation, high levels of bcl-2 protein could protect the cells from undergoing apoptosis in the presence of glucocorticoids and multiple chemotherapeutic drugs.

Taken together, these findings suggest that a high level of bcl-2 protein may play an important role both in lymphoma-genesis and in the development of drug resistance, in follicular, as well as in other varieties of lymphomas. This would provide support for the potential clinical value of bcl-2 protein expression in NHLs, as recently suggested in acute myeloid leukemias and lung carcinoma. However, only few studies have focused on the correlation between bcl-2 expression at the protein level and clinical presentation and outcome in follicular or diffuse lymphomas. This prompted us to analyze the distribution of bcl-2 at the protein level according to histology and phenotype in 290 uniformly treated patients with intermediate or high-grade NHLs enrolled in the LNH87 protocol. To assess its clinical significance and its potential prognostic value, we focused on the correlation between bcl-2 expression, clinical features, and outcome in diffuse large B-cell lymphomas, which is the most frequent and homogeneous immunohistological subgroup of the present series.

From the Départements de Pathologie, Hôpital Henri Mondor, Créteil; Hôpital Saint-Louis, Hôpital Laennec, Hôpital Hôtel Dieu, Paris; Hôpital Dupuytren, Limoges; the Services d’Hématologie clinique, Hôpital Necker, Paris; Hôpital Henri Mondor, Créteil: Université de Liége, Liége, Belgium; Centre Hospitalier Lyon-Sud, Pierre Benite; Hôpital Saint-Louis, Paris; and the Département de Biostatistique et Informatique Medicale, Hôpital Saint-Louis, Paris, France.

Submitted February 9, 1995; accepted August 15, 1995.
Supported in part by grants from C.N.A.M.T.S. and ER 270 from Université Paris-Valde-Marne.
Address reprint requests to Philippe Gaulard, MD, Département de Pathologie, Hôpital Henri Mondor, Créteil cedex, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.


265
MATERIALS AND METHODS

Patients studied for the expression of bcl-2 at the protein level are a subset of the 2,947 patients entered on the LNH87 protocol, a prospective multicentric trial of the Groupe d’Etude des Lymphomes de l’Adulte (GELA), between October 1, 1987 and April 1, 1990.28,30

LNH87 protocol design. Previously untreated adult patients with intermediate or high-grade NHL according to the Working Formulation (WF)31 were stratified into three groups on the basis of age and prognostic factors and were then randomized to receive either ACVBp (adriamycin, cyclophosphamide, vindesine, bleomycin and prednisone), which was the reference induction treatment arm or another alternating anthracyclin-containing regimen. Eligibility criteria was as follows: patients under 70, with biopsy proves intermediate or high-grade histologies (WF, categories D through J). Exclusion criteria included prior treatment, a positive serology to the human immunodeficiency virus, concomitant or previous cancer (except in situ cervix carcinoma or skin epithelioma), heart disease, uncontrolled diabetes mellitus, liver or kidney failure. Patients with a previous history of low-grade NHL or with bone marrow or central nervous system involvement in the setting of Burkitt or lymphoblastic histology were also excluded. A centralized histological review and phenotypic study was strongly encouraged in the LNH87 protocol design. Thus, unstained slides of the initial biopsy specimen and/or were MB2 and CDw75 positive, but did not express MIB1 (CD1c) (30%) positive anaplastic large cell subtype was added to the categories of the WF. Immunophenotypic studies were performed on deparaffinized tissue sections using a panel of monoclonal antibodies directed against B (CD20/L26, CDw75/LN1, MB2), and T (CD3, CD45R0/UCHL1) cell-associated antigens to assess the B- or T-cell lineage of the lymphoma. Lymphomas were considered of B-cell derivation if tumor cells expressed CD20 antigen and/or were MB2 and CDw75 positive, but did not express CD3. They were considered of T-cell derivation if tumor cells expressed CD3 antigen or, if CD3 negative, were CD45R0 positive, and did not express CD20, CDw75 and MB2. Lymphomas were considered as having an undefined phenotype when any other combination was observed.

Patient selection. We chose to analyze bcl-2 protein expression among patients uniformly treated by the ACVBp induction regimen. Among the patients with histologically reviewed NHL who received ACVBp, 348 were selected at random to analyze the distribution of bcl-2 expression according to histology and phenotype. Fifty eight patients were excluded due to inadequate tissue for bcl-2 staining. Histological and phenotypic characteristics of the 290 patients of the bcl-2 protein study group are indicated in Table 1. Among these 290 patients, 151 patients had diffuse large-cell (centroblastic and immunoblastic) lymphoma of B-cell origin, which represented the most important and homogeneous immunohistological subgroup. These were specially analyzed for clinical and diagnostic correlations. Clinical features of these 151 patients and of the remaining 267 eligible patients (ie, with diffuse large B-cell NHL who received ACVBp) of the LNH87 protocol are compared in Table 2. The two groups did not differ for the distribution of either reverse prognostic factors or histological subgroups. Table 3 provides the distribution into the risk groups of the International Prognostic Index32 for the 151 patients studied for bcl-2, as compared with the corresponding patients treated identically during the same period. These data indicate that the patient bcl-2 study group was representative of the patient population with diffuse large B-cell NHL entered on the LNH87 protocol. Thirteen patients in complete response were randomized to receive a high-dose chemotherapy regimen followed by autologous bone marrow transplantation; the remaining 98 good-
cells was statistically more frequent in B-cell lymphomas (109 of 214, 51%) than in T-cell lymphomas (6 of 35, 17%) \((P = .0004)\).

**Bcl-2 expression and clinical features.** Bcl-2 expression according to the clinical characteristics of the patients was studied in the whole group and in the group of patients having diffuse large B-cell lymphoma. For the group having diffuse large-cell lymphoma of B-cell phenotype, the \"high bcl-2 expression\" pattern was more frequent in patients with III-IV stage disease at presentation \((P = .002)\) (Table 5) whereas, in the whole group, this pattern was more frequently associated with nodal presentation \((P = .03)\), bone marrow involvement \((P = .04)\), and III-IV stage disease \((P = .003)\). In contrast, there was no association of bcl-2 expression with the other prognostic factors of the International Index\(^3\); lactate dehydrogenase (LDH), number of extranodal sites, and performance status.

**Bcl-2 expression and outcome in diffuse large B-cell lymphomas.** To evaluate the prognostic significance of bcl-2 protein expression, analysis of outcome was restricted to the patients with diffuse large B-cell lymphomas, because the latter represent the most frequent and homogeneous immunohistological subgroup in the present series. This demonstrated on the 151 patients with diffuse large B-cell lymphoma a significant difference in DFS \((P < .01)\) and survival \((P < .05)\) based on the level of bcl-2 expression (Table 5). Indeed, for patients with high bcl-2 expression, the 3-year estimates of DFS and survival were 60% and 78%, respectively, whereas the 3-year estimates of DFS and survival in the bcl-2 negative/heterogeneous subgroup were 82% and 78% as illustrated in Fig 2A and B. Multivariate analysis showed that only high bcl-2 expression \((P = .01)\) and performance status \((P = .04)\) adversely affected DFS. However, only performance status independently affected overall survival \((P = .002)\).

**DISCUSSION**

In the present report, we have studied the expression of bcl-2 protein in an homogeneously treated group of patients with high-or intermediate-grade of NHL. We show that bcl-2 is expressed by lymphoma cells with a high frequency...
regardless of histologic subgroups, and that high bcl-2 expression is correlated with B-cell phenotype. Furthermore, clinical correlations performed in the more homogeneous immunohistological group of diffuse large B-cell NHL disclose that high bcl-2 expression is correlated with extended stage of the disease and appears to be associated with a poor prognosis. Indeed, DFS and survival were significantly reduced in patients with high bcl-2 expression, although the independent effect of high bcl-2 expression, at the time of analysis, could be demonstrated only on DFS.

To date, most studies of the bcl-2 gene have focused on the presence of the t(14;18), which have been found in the
Table 4. Histological and Immunohistological Characteristics of the 290 Patients Studied for bcl-2 Protein Expression According to the Pattern of bcl-2 Staining

<table>
<thead>
<tr>
<th>bcl-2 Negative/ Heterogeneous</th>
<th>bcl-2 Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td><strong>Histology (WF, Kiel) No</strong></td>
<td></td>
</tr>
<tr>
<td>Diffuse small cleaved, 13</td>
<td>0 (0)</td>
</tr>
<tr>
<td>centrocytic,* 13</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Diffuse mixed,* 22</td>
<td>20 (62)</td>
</tr>
<tr>
<td>Centroblastic centrocytic, 5</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Polymorphic immunocytoma, 10</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Lymphoepithelioid (Lennert's), 6</td>
<td>5 (83)</td>
</tr>
<tr>
<td>Angioimmunoblastic (AILD)-type, 9</td>
<td>9 (90)</td>
</tr>
<tr>
<td>Follicular large cell, 24</td>
<td>10 (42)</td>
</tr>
<tr>
<td>follicular centroblastic, 24</td>
<td>10 (42)</td>
</tr>
<tr>
<td>Diffuse large cell (includes immunoblastic), 183</td>
<td>105 (58)</td>
</tr>
<tr>
<td>Centroblastic, 142</td>
<td>79 (56)</td>
</tr>
<tr>
<td>Immunoblastic B, 9</td>
<td>5 (56)</td>
</tr>
<tr>
<td>Pleomorphic T medium</td>
<td>8 (80)</td>
</tr>
<tr>
<td>and large, 10</td>
<td></td>
</tr>
<tr>
<td>Small non-cleaved (Burkitt), 5</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Burkitt, 5</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Lymphoblastic, 5</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Lymphoblastic (T), 5</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Anaplastic,* 22</td>
<td>15 (68)</td>
</tr>
<tr>
<td>Anaplastic B, 9</td>
<td>2 (22)</td>
</tr>
<tr>
<td>Anaplastic T-nul, 13</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Unclassifiable, 6</td>
<td>4 (80)</td>
</tr>
<tr>
<td>Total 290</td>
<td>159 (55)</td>
</tr>
</tbody>
</table>

Phenotype§

B (n = 214) 105 (49) 109 (51)

T (n = 35) 29 (83) 6 (17)

* Mantle-cell lymphomas.
† 24 cases (2, diffuse mixed; 22, diffuse large cell) were of undetermined phenotype and were not subcategorized according to Kiel.
‡ The KI(CD30) anaplastic large cell subtype was added to the categories of the WF.
§ Not available in 8 cases; 33 (11%) cases were of "null" phenotype.

Majority of follicular lymphomas, and in about 20% of diffuse large cell lymphomas. It has been postulated, although not uncontroversially demonstrated, that the presence of a bcl-2 gene rearrangement or of the translocation t(14;18) seemed to be associated with shorter DFS and/or failure to achieve a complete remission. In contrast, only a few studies have focused on the clinical relevance of bcl-2 expression at the protein level. In the present study, we further extend to a large group of patients previous reports showing that the bcl-2/124 monoclonal antibody provides accurate staining even on routine paraffin-embedded biopsy material. Using this antibody, we confirmed that high expression of bcl-2 is found in a large number of lymphomas with intermediate-or high-grade histology. The apparent discrepancy between the frequency of high expression of the protein and the bcl-2 gene rearrangement is consistent with the findings that bcl-2 protein is expressed in other hematologic malignancies such as acute and chronic myelogeneous leukemia, in a large variety of carcinomas from liver, lung, prostatic gland, as well as in normal tissues, independently of the t(14;18) translocation. These data strongly suggest that, beside the t(14;18) translocation, other pathological mechanisms may increase the expression of bcl-2. As an example, upregulation of bcl-2 expression has already been reported in vitro as a consequence of Epstein-Barr virus (EBV) infection or interleukin-10 stimulation. Alternatively, the transformation process may have occurred in subsets of normal B or T lymphocytes in which constitutive high levels of endogenous bcl-2 protein are present.

This study further confirms previous observations that most high-grade mucosa-associated lymphoid tissue (MALT) lymphomas are bcl-2 negative, as the latter pattern was shown in 12 of the 14 large B-cell lymphomas of the gastrointestinal tract (data not shown). The absence of detectable bcl-2 protein in a number of intermediate- and high-grade lymphoma, especially in high-grade MALT lymphomas, might reflect a breakdown in bcl-2 gene expression at the transcriptional or the posttranscriptional level. However, whatever the mechanism of deregulation of the bcl-2 gene, the finding of high expression of bcl-2 observed in a large proportion of NHLs raises the question of its clinical value and biological significance, because bcl-2 is now regarded as a member of a new category of oncogenes involved in blocking programmed cell death, thus leading to abnormal accumulation of cells.

Our analysis, which was performed on fixed paraffin-embedded material, thus preserving morphology, easily identified three patterns of bcl-2 protein expression. We chose to individualize the group with virtually all tumor cells bcl-2 positive from the other two groups exhibiting either no or intermediate bcl-2 expression, because the labeling in the high bcl-2 expression group was homogeneous and easily distinguishable from the other two. In addition, several recent in vitro and in vivo studies have pointed out the physiological and clinical importance of high bcl-2 expression. Furthermore, when comparing, in the present study, survival according to the level of bcl-2 expression, no difference was observed for the bcl-2 negative and bcl-2 intermediate groups. In the present series, including patients who were all treated with the same induction regimen of the LNH87 protocol containing high doses of anthracyclins and cyclophosphamide, we show that the patients' outcome after chemotherapy was predicted by bcl-2 expres-

Table 5. Clinical Features of the 151 Studied Patients With Diffuse Large B-Cell Lymphomas According to bcl-2 Expression

<table>
<thead>
<tr>
<th>bcl-2 Positive (n = 64)</th>
<th>bcl-2 Negative/Heterogeneous (n = 87)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%)</td>
<td>No. (%)</td>
</tr>
<tr>
<td>Age (&gt;60 yr)</td>
<td>25 (39)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>36 (56)</td>
</tr>
<tr>
<td>Stage III-IV</td>
<td>48 (72)</td>
</tr>
<tr>
<td>Performance status (&gt;2)</td>
<td>18 (25)</td>
</tr>
<tr>
<td>LDH &gt; 1N</td>
<td>43 (65)</td>
</tr>
<tr>
<td>Extraneural sites (&gt;1)</td>
<td>12 (18)</td>
</tr>
<tr>
<td>3-yr DFS (%)</td>
<td>80 ± 14</td>
</tr>
<tr>
<td>3-yr survival (%)</td>
<td>61 ± 12</td>
</tr>
</tbody>
</table>
Reed22,23,44 who reported that bcl-2 transfected murine and human lymphoid cell lines exhibit higher resistance to several antineoplastic agents, including those used in the present study. Indeed, in bcl-2 transfected cells, chemotherapy induces arrest of proliferation, but unlike in control cells, death by apoptosis is prevented, and drug withdrawal results in reinitiation of cell growth.

In the more homogeneous subset of diffuse large B-cell lymphomas, high bcl-2 expression appears, in multivariate analysis, as an independent prognostic factor for DFS together with performance status. Therefore, it is not likely that the association of high bcl-2 expression with a poor clinical outcome is due to its correlation with stage III-IV disease. Moreover, the fact that high bcl-2 expression is not correlated with the level of LDH, suggests that the poor outcome is mainly due to delayed cell death or resistance to treatment, but not to increased cell proliferation. This hypothesis is consistent with the putative role of bcl-2 in lymphomagenesis, as a suppressor of apoptosis without effect on cell proliferation.46 In contrast, the finding that high bcl-2 protein expression does not appear to affect independently overall survival of patients with diffuse large B-cell lymphomas may be due to an insufficient follow up, and thus should be reevaluated later.

In conclusion, in this series of uniformly treated patients with a diffuse large B-cell lymphoma, high bcl-2 protein expression was closely linked to reduced DFS and, at a lesser extent, to shorter survival. Together with the results of recent studies,26,27,45 performed on a smaller number of patients and/or receiving heterogeneous treatments, it appears that bcl-2 protein expression, as evaluated in routinely paraffin-embedded tissue, may be considered as a new independent biological
prognostic parameter. The bcl-2 protein expression, like the Ki67 proliferating index, the presence of the lymphocyte homing receptor (CD44), or deregulation of the p53 oncogene may aid in the identification of patient risk groups.

ACKNOWLEDGMENT


REFERENCES

3. Cleary ML, Sklar J: Nucleotide sequence of a (14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint cluster region near a transcriptionally active locus on chromosome 18. Proc Natl Acad Sci USA 82:7439, 1985
23. Miayashita T, Reed JC: Bcl-2 gene transfer increases relative resistance of S49.1 and WEHI72 lymphoid cells to cell death and DNA fragmentation induced by glucocorticosteroids and multiple chemotherapeutic drugs. Cancer Res 52:5407, 1992
with chemotherapy alone in the LNH-87 protocol group I. A GELA study. Fifth International Conference on Malignant Lymphoma, June 1993, p 98 (abstr)


