ABSTRACT

Although B-cell diffuse large-cell lymphoma (DLCL) can respond to chemotherapy and radiotherapy, a large number of patients are still resistant to treatment. Caspase-3 is an enzyme crucial to the apoptotic process and may be important in the clinical outcome of these patients. The pattern of caspase-3 expression was studied in 54 cases of DLCL using immunohistochemistry and quantitative reverse-transcription PCR. Tumor cells displayed both a diffuse cytosolic and a punctate cytosolic staining for caspase-3. Kaplan-Meier survival curves indicated that tumor cells with a diffuse cytosolic expression of caspase-3 correlated with a poor prognosis ($P > 0.0004$). In addition, a punctate cellular localization was associated with complete response to treatment ($P = 0.011$). Cases with a small percentage of lymphoma cells expressing caspase-3 also tended to show poor survival ($P > 0.09$). Levels of caspase-3 mRNA were not significant ($P > 0.17$), although a weak trend was observed similar to the immunohistochemical analysis. The pattern of expression of caspase-3 was also assessed with respect to terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) positivity in both reactive lymph nodes and B-cell DLCL cases. Our results suggest that TUNEL-negative cells are not caspase-3-positive and that there is no correlation between DLCL cases with a high degree of DNA fragmentation and caspase-3 immunostaining. Furthermore, a survival curve indicated that a high TUNEL positivity was associated with a poor survival probability ($P < 0.02$) and a poor response to treatment ($P = 0.04$). These results confirm the dynamic nature of caspase-3 expression in DLCL and suggest that the pattern of expression of the enzyme has prognostic significance.

INTRODUCTION

The heterogeneous nature of B-cell DLCL has often made the basis of classification very difficult and is believed to be the major reason for the variation in clinical outcome that has been reported (1, 2). Although these tumors can be treated successfully with chemotherapy or radiotherapy, primary chemoresistance and relapse often occur and are the major causes of death in these patients. Most importantly, tumors that relapse frequently are resistant not just to the initial agent used for treatment but also to other unrelated compounds.

Recent evidence suggests that most agents of cancer chemotherapy used to treat tumors induce apoptosis in their target cells (2, 3). Apoptosis describes a distinct form of cell death that occurs in most mammalian tissues and is crucial for the development and homeostasis of eukaryotic organisms. In this process, a family of cysteine proteases called caspases are activated and cleave key substrates during apoptosis, such as the inhibition of topoisomerase (13). Crystalline analysis revealed that these two fragments assemble to cleavage of an aspartate residue of the 32-kDa inactive caspase-3 zymogen produces two active fragments of 17–21 and 10–12 kDa. Many hematopoietic malignancies display a multidrug-resistance phenotype, and this is thought to be due in part to the overexpression of proteins such as P-glycoprotein, glutathione S-transferase, and members of the multidrug resistance-associated protein family (14–16). However, differences in primary and secondary chemoresistance do not appear to be solely attributable to the expression of these proteins. Emerging evidence suggests that many tumor cells, including DLCL cells, are intrinsically unable to activate the apoptotic machinery and may, therefore, be fundamentally resistant to chemotherapy and, possibly, radiotherapy (3).

The expression of caspase-3 in a variety of non-Hodgkin’s lymphomas and Hodgkin’s disease has already been reported (17–19) and found to be highly variable. Recent evidence, however, suggests that the level of expression and the localization of this enzyme may have significance with respect to the progression of tumorigenesis (20). We therefore investigated the expression of caspase-3 using immunohistochemistry and a quantitative RT-PCR technique in DLCL patients prior to treatment. In addition, we estimated the rates of apoptosis as measured by DNA fragmentation and examined these data with respect to the expression of caspase-3. Finally, we correlated all data with clinical outcome, including stage at presentation, response to therapy, and survival.

Our results show that there is a high level of variation in the immunostaining and pattern of expression of caspase-3 in DLCL and that this is not related to the level of DNA fragmentation as measured by the TUNEL assay. A diffuse cytosolic localization of caspase-3 in tumor cells was a very poor prognostic factor, whereas an intense punctate expression indicated a higher survival probability. Finally, our results also show that a high level of TUNEL staining, and less significantly, patients with a low percentage of tumor cells expressing caspase-3, also predict a poor outcome for patients with B-cell DLCL.

MATERIALS AND METHODS

Patients. Fifty-four consecutive cases of high grade DLCL tissue biopsies taken for routine diagnosis with patient consent and ethical permission were retrieved from the Department of Pathology archives at Leicester Royal Infirmary. These cases were diagnosed as high-grade B-cell lymphomas by an experienced histopathologist and confirmed by a standard panel of lymphoma antibodies. The ages of the patients ranged from 18 to 85 years, with a median of 69 years and a mean of 65 years. The cases were diagnosed between 1991 and 1996, and analysis was carried out in April 1998. The vast majority of patients were treated with a CHOP (cyclophosphamide, Adriamycin, vincristine, and prednisone) regime, and in some cases this was combined with radiotherapy. Staging of the tumors at presentation was carried out according to Ann Arbor (21). Complete remission was defined as absence of clinically detectable disease for 6 months after completion of treatment, and partial...
remission was defined as a reduction in disease bulk short of 50% as measured by computed tomography scanning. All patients presented without prior treatment.

**Immunohistochemistry.** A standard avidin-biotin complex method with alkaline phosphatase detection was carried out. Formalin-fixed paraffin-embedded sections were dewaxed in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 20 min in 0.01 M sodium citrate buffer (pH 6.0). The primary antibody to caspase-3 (Transduction Laboratories, Lexington, KY) was applied at a dilution of 1:1000 and incubated overnight at 4°C. After incubation, the slides were treated with biotinylated rabbit antimonoclonal immunoglobulin (1:600 for 30 min; Dako Ltd., Ely, UK) washed as before, and then treated with streptavidin and biotinylated alkaline phosphatase according to the manufacturer’s instructions (Dako). The slides were then washed, and the signal was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. A negative control reaction with no primary antibody was always carried out alongside the reaction containing sample. The specificity of the caspase-3 antibody was confirmed by comparison with control antibodies.

**TUNEL Staining.** TUNEL staining was carried out as described previously (22). Tissue sections were dewaxed as detailed earlier and then treated with streptavidin and biotinylated alkaline phosphatase according to the manufacturer’s instructions (Dako). The slides were then washed, and the signal was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as described above.

**RT-PCR ELISA.** Measurement of gene expression of caspase-3 by RT-PCR ELISA was carried out as outlined earlier (23). A 10-μm slice of each tumor tissue section was cut and resuspended in 100 μl of lysis/bind buffer. The tissue was incubated with 50 μg/ml proteinase K for 1 h at 37°C. The lysate was then centrifuged for 30 s at 10,000 g, and the supernatant was mixed with oligo(dT)-linked Dynabeads (Dynal Ltd., Wirral, UK). The mRNA was extracted, and cDNA was synthesized with controls for genomic DNA contamination as described previously (23). The cDNA-linked beads were then resuspended in PCR buffer (45 mM Tris (pH 8.8), 11 mM (NH4)2SO4, 4.5 mM MgCl2, 200 μM dNTPs, 110 μg/ml ultrapure BSA (Advanced Protein Products Ltd., Brierley Hill, UK), 6.7 mM β-mercaptoethanol, 4.4 μM EDTA (pH 8.0)), containing 10 pmol of forward and 10 pmol of reverse primer. The following primers were used for amplification of the caspase-3 and GAPDH genes:

- **Forward caspase-3 primer:** 5′-CACAATTTTTTCAGAGGGATGC-3′
- **Reverse caspase-3 primer:** 5′-GCTACATTCTGCGATGGCC-3′
- **Forward GAPDH primer:** 5′-AGAAGATCATCCTCCGTGCT-3′
- **Reverse GAPDH primer:** 5′-GCAATGTCGGTTTCAATAC-3′

A “hot-start” PCR was then carried out as follows: one cycle of denaturation at 98°C for 3 min, holding at 60°C during addition of 1 U of Taq DNA polymerase (Life Technologies Ltd., Paisley, Scotland); primer extension at 72°C for 30 s; 4 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and primer extension at 72°C for 30 s; 23 cycles and 30 cycles for GAPDH and caspase-3, respectively, of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and primer extension at 72°C for 30 s. Preliminary experiments were carried out to confirm that the respective number of cycles maintains the amplification reaction in exponential phase. All PCR products were then visualized using agarose gel electrophoresis. The predicted sizes of the amplified products of caspase-3 and GAPDH were 272 and 354 bp, respectively.

An ELISA system was used to quantify the RT-PCR products. To enable the capture of forward strand cDNA during the assay all forward primers were synthesized 5′-biotinylated. Covalink plates were first biotinylated and then treated with avidin. After washing and blocking, the wells were then treated with 0.25 M NaOH to denature the PCR products and allow the capture of biotinylated forward strands. The probe used for caspase-3 hybridization was 5′-CAATGGCCAGTCAGTTTCC-3′, and the probe used for GAPDH hybridization was 5′-GTGTAAGGACAAGGAGCC-3′. Both probes were labeled with digoxigenin as described previously (23). After incubation with oligonucleotide probes, the plates were washed and treated with alkaline phosphatase-conjugated antidigoxigenin. After washing, the plates were incubated in 1 mg/ml p-nitrophenyl phosphate in 1 M diethanolamine (pH 9.8), for 2.5 h at 37°C. The samples were then read at 405 nm with a differential of 630 nm on a WellScan Multiwell Plate Reader (Denley Instruments Ltd., England). All ELISA measurements carried out in duplicate. Controls for nonspecific binding of probes and plate quality were also included.

**Scoring Methods.** Scoring was carried out blinded to the outcome status of the patient. The immunohistochemical and TUNEL results were reviewed by two independent observers (S. D. and H. S. B.). The percentage of tumor cells with immunostaining was graded as follows: 1 (0–25%), 2 (26–50%), 3 (51–75%), or 4 (76–100%). The number of positive nuclei detected by the TUNEL assay was graded as 0 (0–1), + (2–5), ++ (6–10), or ++++ (>10). The expression of caspase-3 was graded as diffuse cytosolic or punctate cytosolic if >50% of tumor cells staining showed one of these patterns of localization. All scoring methods were measured in four random fields using a ×40 objective.

**Statistics.** Variables associated with caspase-3 immunostaining and immunohistochemical localization, RT-PCR data, and TUNEL positivity were analyzed by the χ2 test and the Kruskal-Wallis test. The survival curves were plotted according to the Kaplan-Meier procedure and confirmed by a log-rank test using the statistical package SPSS. *P* < 0.05 was considered statistically significant.

**RESULTS**

**Immunohistochemical Analysis of Caspase-3 in Lymph Node and B-Cell DLCL.** The results of the caspase-3 immunostaining for lymph node tissue are shown in Fig. 1. The majority of the cells within

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Fig. 1. Photomicrographs showing immunohistochemical staining of caspase-3 (A) and DNA fragmentation by TUNEL staining (B) in reactive tonsil tissue. The low power photomicrograph (A) shows that the majority of cells in the germinal center are caspase-3 immunopositive with a diffuse cytosolic pattern. Selective cells within the center are more intense with a higher immunopositivity. Fewer cells in the interfollicular region are immunopositive; these appear to be plasma cells and immunoblasts. The tingible body macrophages are not caspase-3 immunopositive but show strong TUNEL staining in the high power photomicrograph (B).
the germinal center are positive and appear to have a homogeneous diffuse cytosolic staining. Fewer cells outside the germinal center are positive for staining; however, most of these appear to be plasma cells and immunoblasts. This immunolocalization pattern confirms that reported earlier (17). In addition, there is a population of immunopositive cells within the germinal center that have a more intense staining. The majority of the staining cells in the interfollicular region also appear to be more intensely stained.

A marked difference in the pattern of expression of caspase-3 was observed in the lymph nodes of DLCL patients (see Fig. 2). Half of the cases appeared to have the diffuse cytosolic staining for caspase-3 (53.7%) similar to that observed within the germinal center of lymph nodes. Of the 54 cases, only 5 appeared to have a highly mixed pattern of staining. There was also a large variation in the number of tumor cells with positive immunostaining for caspase-3, with the majority of cells >50% positive (70.3%). At least two of the cases appeared to

![Fig. 2. Photomicrographs showing immunohistochemical staining of caspase-3 (A, B, D, E) and DNA fragmentation by TUNEL staining (C, F) in DLCL cases. A case showing diffuse cytosolic staining for caspase-3 is shown in low power (A) and high power (B) photomicrographs and the corresponding TUNEL staining at low power (C). Diffuse cytosolic staining was associated with a high incidence of tingible body macrophages and cells showing DNA fragmentation as measured by TUNEL. A second case illustrates punctate cytosolic staining for caspase-3 (arrows) in low power (D) and high power (E) photomicrographs and the corresponding TUNEL staining at low power (F). Punctate cytosolic staining was associated with reduced DNA fragmentation and few tingible body macrophages.]
have no tumor cells positive for caspase-3 whatsoever. There did appear to be variation in intensity of staining between cases; however, this could not be quantified using this technique.

**TUNEL Staining in B-Cell DLCL.** The majority of cases showed a significant number of cells with DNA fragmentation and an apoptotic count (++; +++) as assessed by TUNEL staining (59.3%). Both apoptotic bodies and tingible body macrophages could be observed throughout the tissue. In addition, a significant number of the cases had very little evidence of TUNEL staining (29.6%), including the two cases observed earlier with very few tumor cells immunopositive for caspase-3. As shown in Fig. 1, it would appear that the tingible body macrophages containing apoptotic bodies within the germinal center were not caspase-3 immunopositive. It was difficult to assess, however, whether isolated apoptotic bodies were positive.

**RT-PCR ELISA.** Only 42 cases of the 54 in the archive were analyzed by quantitative RT-PCR for caspase-3 expression because the quality of the mRNA extracted in the other specimens was poorly preserved. Fig. 3 shows an agarose gel illustrating the range of the quality of the mRNA extracted in the other specimens was poorly analyzed by quantitative RT-PCR for caspase-3.

**Correlation Between Caspase-3 Immunohistochemical Analysis and Clinicopathological Factors.** As shown in Table 1, χ² tests showed that there was no correlation with age, sex, or stage and the pattern of expression and immunostaining of caspase-3. However, a punctate pattern of staining correlated with a complete response to treatment versus partial/no response (P = 0.011). In addition, this type of staining was associated with a very high probability that the patient was alive at the end of the study (P < 0.001). Furthermore, a low TUNEL positivity was weakly associated with a complete response versus no response (P = 0.117). This pattern of staining also correlated with a high probability that the patient was alive at the end of the study (P = 0.01). The gel shows minimal variation in GAPDH levels between the two groups 1–5 (≥1) and 6–10 (<1); however, the variation in the levels of caspase-3 mRNA is striking. A weak trend was observed when the gene expression of caspase-3 as measured by this assay was correlated with the immunostaining data (P = 0.160, Kruskal-Wallis; results not shown).

**Survival Analysis.** Fig. 4 shows the log-rank statistic and survival curves for caspase-3 pattern of staining, immunostaining, gene expression, and TUNEL positivity. Patients with tumor cells expressing diffuse cytosolic immunostaining for caspase-3 had a poor prognosis when compared with those expressing a punctate staining (P > 0.0004 log-rank). Furthermore, a low percentage of cells with positive immunostaining showed a borderline association with poor survival (P > 0.09). Measurement of gene expression by a quantitative RT-PCR ELISA method did not show a significant association with survival probability (P > 0.17), although a trend was clearly present in that a low caspase-3 gene expression was associated with a poor prognosis. In addition, patients with a high TUNEL positivity had a low survival probability (P < 0.02).

Other statistical analyses indicated that patients presenting with a stage III or IV disease tended to have a poor survival probability (P = 0.018, χ²; P = 0.069, Kruskal-Wallis; data not shown). Furthermore, if the patient was >66 years of age at diagnosis, his or her survival probability also tended to be quite poor (P = 0.009, Kruskal-Wallis; data not shown).

**DISCUSSION**

The immunolocalization of caspase-3 staining in reactive lymph node is similar to that reported earlier (18, 19). That is, homogenous diffuse cytosolic immunostaining was present in the germinal center cells of reactive follicles, with lesser staining in the mantle zone and interfollicular regions. With respect to the immunohistochemical localization of the enzyme, early reports indicated cytosolic and occasional nuclear staining but no punctate staining (18). More recent reports (24, 25), however, proposed that the precursor form of caspase-3 has both a mitochondrial and a cytosolic distribution. Further analysis will be required to confirm this hypothesis.

Table 1 Correlation between clinicopathological factors and immunohistochemical localization, percentage of immunostaining, and quantitative RT-PCR analysis for caspase-3 mRNA and TUNEL positivity.

<table>
<thead>
<tr>
<th>Immunohistochemical punctate localization</th>
<th>High percentage of immunostaining (GAPDH relative)</th>
<th>High levels of caspase-3 mRNA</th>
<th>Low TUNEL positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) △ &lt;66 vs. ≥66</td>
<td>0.510</td>
<td>0.357</td>
<td>0.789</td>
</tr>
<tr>
<td>Sex (female)</td>
<td>0.113</td>
<td>0.505</td>
<td>0.555</td>
</tr>
<tr>
<td>Stage I and II</td>
<td>0.838</td>
<td>0.744</td>
<td>0.235</td>
</tr>
<tr>
<td>Complete response</td>
<td>0.011</td>
<td>0.572</td>
<td>0.235</td>
</tr>
<tr>
<td>Alive status &lt;0.001</td>
<td>0.623</td>
<td>0.13</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* mRNA was successfully extracted from 42 of the 54 sections.

△ The median age of the cohort was 66 years.

* Stage information was available for only 48 cases. Analysis of histological stage I and II versus III and IV.

* No treatment was given to 8 patients, and response information was available for only 40 patients; analysis was made of no response and partial response versus complete response.

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versus 50% appears to confirm earlier reports (18). In addition, the gene expression of caspase-3 may be associated with the mitochondrial localization of procaspase-3. Moreover, the immature form of the enzyme has been detected in the cytosol and the mitochondria of various tissues by protein fractionation (26). Therefore, the punctate staining described in this report may be associated with the mitochondrial localization of procaspase-3.

The variation on protein expression of caspase-3 in DLCL again appears to confirm earlier reports (18). In addition, the gene expression of caspase-3 was highly variable according to the quantitative RT-PCR results. The mRNA and protein values did show a weak association. However, the lack of a highly significant correlation may be due to a number of reasons. First, the immunohistochemical analysis carried out did not accurately evaluate the intensity of caspase-3 protein expression, just the percentage of tumor cells with positive immunostaining. Second, the quantitative RT-PCR assay indiscriminately quantifies caspase-3 gene expression levels within a tissue section. This usually contains a mixture of background cells, some of which express caspase-3, along with tumor cells.

With the DLCL specimens, the immunostaining data almost reached significance in that the greater the percentage of tumor cells expressing caspase-3, the better the prognosis for the patient. A weak trend was also observed with the quantitative RT-PCR data in that low caspase-3 mRNA expression was associated with a poor prognosis, although this was not statistically significant. Overexpression of caspase-3 has in the past been associated with an increased sensitivity to therapy (20), and a recent report indicated that those childhood neuroblastomas that can spontaneously regress also had high levels of caspase-3. Other researchers have implicated low levels of caspase-3 in resistance to chemotherapy- and radiotherapy-induced apoptosis in cell lines (27).

Nevertheless, there were cases of large-cell lymphomas that had little or no staining for caspase-3 yet survived significantly greater than the median length of time. It is possible that these tumor cells used different caspases to effect programmed cell death. The two cases of DLCL with no caspase-3 tumor staining whatsoever also had no DNA fragmentation as detected by the TUNEL assay. This would appear to confirm the earlier report (28) of caspase-3 being essential for the nuclear changes associated with apoptosis in mouse embryonic stem cells and fibroblasts.

Large B-cell lymphomas cases with intense punctate cytosolic caspase-3 staining tended to have a better prognosis than patients with diffuse cytosolic staining, and these results were highly significant. The immunohistochemical localization of the enzyme was more important than the immunopositivity with respect to survival and again emphasized the concept of redundancy within the apoptotic program. The exact localization of the active and inactive enzyme in a cell, and its importance with respect to the apoptotic process, is still uncertain as indicated above. Many of the early substrates discovered for caspase-3 were associated with the nucleus; hence, it was initially assumed that this protease translocated into the nucleus at some stage during the apoptotic process. This view was also reinforced by some researchers describing occasional nuclear staining (18, 20). For example, the translocation of caspase-1 and -3 to the nucleus has been associated with neuroblastomas susceptible to regression (20). Recent evidence, however, indicates that caspase-3 is capable of activating a cytosolic protein that translocates into the nucleus and degrades chromatin DNA during apoptosis (29, 30). One report also suggested that this nuclear staining colocalized with TUNEL staining and apoptotic cells (20). The issue is controversial because an earlier report suggested most cells positive for caspase-3 were not TUNEL positive (18). Certainly our results also indicate that in the germinal center TUNEL-positive cells are not caspase-3 immunopositive.

Furthermore, cases with diffuse cytosolic staining are associated with a high degree of TUNEL staining. Many of the these tumor cases exhibited an apoptotic pattern very similar to that seen in reactive lymph nodes, i.e., a diffuse localization of caspase-3 and a high degree of DNA fragmentation. Patients exhibiting this pattern of expression tended to have a poor prognosis. There are a number of reasons why this may occur. Mancini et al. (24) suggested that the separate forms of procaspase-3 (cytosolic and mitochondrial) indicated the existence of distinct activation pathways. The diffuse cytosolic staining exhibited in the germinal centers of secondary follicles may indicate sensitization to a particular form of receptor-mediated cell death, whereas tumor cells expressing a punctate immunohistochemical localization may be sensitized to a different form of cell death, such as chemotherapy or radiotherapy.

In addition, the high level DNA fragmentation detected by the TUNEL assay probably indicates a higher proliferative rate. Gisbertz et al. (31) recently reported a correlation between high proliferation and high apoptotic rates in DLCL. This correlation was not seen in low grade lymphomas. Other authors have also shown a correlation between apoptotic and proliferative indices in malignant non-Hodgkin’s lymphomas (32).

Finally, the ability of tumor cells to avoid immune surveillance has been highlighted recently. It has been demonstrated that a variety of lymphoma cells express Fas ligand and are capable of inducing cell death in CTLs (33). Furthermore, these tumor cells appeared to have reduced sensitivity to Fas ligand-induced cell death. Our own data (results not shown) and other observations confirm that DLCL cells can express high levels of Fas ligand. It remains to be determined whether this contributes to the poor prognosis associated with a high level of DNA fragmentation as determined by TUNEL.

We have shown that the pattern of expression of caspase-3 immunostaining is highly variable and has significance for prognosis in DLCL. Furthermore, the importance of this enzyme in the apoptotic process seems to be highly complex. Future experiments will focus on...
the importance of the localization of the enzyme with respect to the different signaling pathways used to induce cell death.

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Immunohistochemical Localization of Caspase-3 Correlates with Clinical Outcome in B-Cell Diffuse Large-Cell Lymphoma


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