Expression of BAX in Plasma Cell Dyscrasias

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ABSTRACT
Several studies demonstrate that the BCL-2 and BCL-XL antiapoptotic genes are variably expressed in plasma cells of patients with multiple myeloma (MM). However, the plasma cell expression of BAX protein, their major proapoptotic partner, has not been investigated. Our initial Western blot analysis of myeloma cell extracts also suggested patient variability in the expression of BAX, which was not altered by exposure to interleukin 6. To further investigate the significance of BAX expression, we performed immunohistochemistry on archival bone marrow biopsies and compared BAX staining to BCL-2 immunostaining. Expression was first evaluated in 104 patients with reactive plasmacytosis, monoclonal gammopathy of undetermined significance/smoldering MM, or active MM. An increase (P < 0.05) in expression of both BAX and BCL-2 was detected in MM patients compared with patients with reactive plasmacytosis. Patients with monoclonal gammopathy of undetermined significance/smoldering MM had intermediate values. For correlations with outcome, expression was assessed in 43 patients at diagnosis who were treated with melphalan and prednisone; 30 at diagnosis who were treated with vincristine, Adriamycin, and dexamethasone; and 29 at relapse who were treated with second-line therapy. There was no correlation between BAX or BCL-2 expression and response to chemotherapy or duration of response or between BCL-2 expression and survival. However, patients who demonstrated extremely low plasma cell BAX expression had significantly increased survival. This was true for patients initially treated with melphalan and prednisone or vincristine, Adriamycin, and dexamethasone, as well as patients studied at relapse. BAX expression did not correlate with expression of proliferating cell nuclear antigen used as a marker of proliferation. These data indicate a myeloma-specific increase in BAX expression in plasma cells and suggest that low BAX expression identifies a cohort of patients with long survival, which is not specifically associated with low proliferating cell nuclear antigen expression.

INTRODUCTION
Although the antiapoptotic BCL-2 gene was first discovered because of its dysregulated expression in B-cell lymphomas, high BCL-2 protein levels have been detected in many other types of malignancies (1). Because an antiapoptotic protein should theoretically prevent chemotherapy-induced tumor cell death, correlations between BCL-2 expression and poor responses to therapy with shorter survival would be anticipated. Indeed, several studies (2, 3) have documented such a correlation in certain tumor models. However, in other neoplasms, expression is not correlated with a poor prognosis and, in contrast, may be associated with a favorable outcome (4, 5). Thus, the role of BCL-2 in the clinical course of cancer patients is still unclear and is probably tumor type specific. One possible reason for disparate results in different tumor models is that other members of the BCL-2 protein family also significantly modulate cell death, and their altered expression in certain tumors may be more important in determining prognosis. One of these may be BAX protein, the major proapoptotic binding partner of BCL-2. Studies in metastatic breast cancer and chronic lymphocytic leukemia (6, 7) confirm that BAX expression may influence outcome.

Several studies have demonstrated significant expression of BCL-2 and BCL-XL in malignant plasma cells of patients with MM (8–15). Although several small studies have shown that BCL-2 expression does not correlate with decreased responsiveness to therapy (9, 10), a recent investigation (16) has demonstrated that it inversely correlates with plasma cell proliferative potential, suggesting it may influence survival independent of chemoresponsiveness. Furthermore, BCL-XL expression is associated with chemoresistance (14). Because BAX is a major dimerization partner of both BCL-2 and BCL-XL and counters their function, it may also influence clinical outcome. BAX is also expressed in MM cell lines (17). We, thus, evaluated expression of that proapoptotic protein in myeloma specimens and compared it with BCL-2 expression. Our results indicate a malignancy-specific increase in expression of BAX as...
well as BCL-2 proteins. Expression of either protein did not correlate with responsiveness to therapy, but in a small cohort of patients whose tumor cells exhibited low BAX expression, long survival was observed.

PATIENTS AND METHODS

Patient Population. Archival BM biopsies obtained from patients at the West Los Angeles Veterans Affairs and Kaiser Permanente (Woodland Hills, CA) Hospitals were immunostained for BCL-2 and BAX expression. These patients were all biopsied between 1989 and 1996. Patients from Kaiser Hospital were all biopsied at initial presentation, had a diagnosis of active symptomatic MM made clinically and pathologically, and were started on M&P as induction chemotherapy. Patients received repeated cycles every 4–6 weeks, and those who responded were continued on treatment for at least 1 year. Those patients who responded but had continued presence of a paraprotein received therapy until relapse. In a few patients whose paraproteins disappeared, chemotherapy was discontinued. Eighty-five % of patients received at least 80% of the planned dose of chemotherapy.

The immunostained BMs from the West Los Angeles Veterans Affairs Hospital were obtained from patients who had one of four different diagnoses: reactive polyclonal plasmacytosis secondary to infectious or inflammatory disorders; MGUS/smoldering myeloma; symptomatic myeloma at diagnosis; or symptomatic myeloma at relapse. Patients with myeloma at diagnosis were started on VAD receiving cycles every 4 weeks. Patients who responded were maintained on treatment for at least 1 year. Every other cycle contained 4 instead of 12 days of high-dose dexamethasone. In responding patients with continued presence of paraprotein, VAD was continued until relapse, whereas asymptomatic responding patients, who demonstrated disappearance of paraprotein, had therapy ceased and were followed until relapse. Patients who reached a cumulative dose of Adriamycin between 450 and 550 mg/m² had that drug terminated. Eighty % of patients received at least 80% of the planned dose of VAD.

Patients with myeloma at relapse had initially responded to VAD and subsequently demonstrated clinical relapse at the time of biopsy. After BM biopsy, they were retreated with either M&P, high-dose dexamethasone alone, or other regimens.

Active myeloma was defined in symptomatic patients according to the Myeloma Task Force (18). Patients with MGUS were diagnosed according to the criteria of Kyle et al. (19). They have been followed for a mean of 4 years without evolution to myeloma. Patients with smoldering myeloma were defined according to prior criteria (20).

Isolation of Fresh Myeloma Cells. After Ficoll-Hypaque separation of BM cells, myeloma cells were further separated on an immunoaffresorption column as described previously (21), using biotinylated anti-CD38 antibody to isolate high CD38-expressing cells.

Western Blot. As described previously (22), cells were lysed in lysis buffer, and lysates were cleared at 14,000 rpm for 15 min at 4°C. Proteins were separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were then incubated with rabbit antihuman BAX anti-body (from Dr. J Reed, Burnham Institute, La Jolla, CA) for 1 h. After four washes, the membranes were overlaid with 1 μg/ml horseradish peroxidase-labeled goat antirabbit IgG antibody (Amersham), and protein bands were detected with an enhanced chemiluminescence system.

HIC. BM biopsies obtained at the West Los Angeles Veterans Affairs Hospital were fixed in formalin, and those obtained from Kaiser Hospital were fixed in Bouin’s solution. HIC was performed as reported previously (6, 23) using a microwave antigen retrieval method and biotinylated goat antirabbit IgG, followed by an avidin-biotin complex reagent (Vector Laboratories, Inc.) with horseradish peroxidase. Colorimetric detection of bound antibody was achieved with diaminobenzidine, followed by counterstaining with hematoxylin. Expression of BAX or BCL-2 was graded on an arbitrary scale and designated 0, 1+, 2+, 3+, or 4+. Determination of frequency of expression used the following guidelines: 0, 0% staining of plasma cells; 1+, 1–10% of cells; 2+, 11–50% of cells; 3+, 51–90% of cells; and 4+, 91–100% of cells. Intensity of staining was similarly graded in an arbitrary fashion with scores of 0–4+. Stained specimens were examined in blinded fashion by two of the authors (S. R. and A. L.), and each observer initially independently assessed expression. In 94% of cases, identical immunopositive frequency scores were initially obtained by the two observers. In 87% of cases, identical intensity scores were initially obtained. After further examination and discussion, agreement was reached as to the degree of expression in all cases. Negative controls consisted of simultaneously stained preparations, in which preimmune antisera was used instead of the BCL-2/BAX antibody, or the antibodies were specifically preabsorbed with specific BCL-2 or BAX peptide. These controls were uniformly negative for immunostaining. BCL-2-expressing BM lymphocytes, and BAX-expressing erythroid and myeloid precursors served as internal positive controls. The anti-BAX and anti-BCL-2 antibodies were generated as described previously (6, 23). The anti-BAX antibody was specific for a peptide corresponding to amino acids 43–61 of the human BAX protein. The anti-BCL-2 antibody was specific for amino acids 41–54 of human BCL-2 protein. Frequency of PCNA expression was the percentage of positive-staining plasma cells, determined by counting at least 250 plasma cells from three different areas in BMs from myeloma patients. For MGUS patients, only 50–100 plasma cells were enumerated to determine frequency of PCNA staining. Small numbers of PCNA-expressing immature normoblasts and WBC precursors served as internal positive controls. The anti-PCNA antibody was monoclonal antibody PC-10, purchased from DAKOpatts (Copenhagen, Denmark). These studies were approved by Human Subject Protection Committees of the West Los Angeles Veterans Affairs and Kaiser Permanente Hospitals in accord with assurances filed with and approved by the Department of Health and Human Services.

Clinical Information. Clinical characteristics, response to therapy, duration of response, and survival were determined by chart review. Response was determined by examination of follow-up protein electrophoretic studies 4–9 months after initiation of chemotherapy. For the purposes of this study, response was defined as at least a 50% reduction in serum paraprotein or a 75% reduction in urine paraprotein for patients with light...
chain myeloma. These reductions in M protein had to last for at least 3 months to be considered a response. For a few patients with nonsecreting myeloma, response was defined as a decrease in plasma cell marrow infiltration to <5% on repeat BM examination. Response duration for responders was calculated from the date of initiation of therapy until the date of documented recurrence. Survival was calculated from the date of BM biopsy until death or last follow-up examination. Survival and response duration data were censored as of August 1, 1997.

Statistics. Differences in BCL-2/BAX expression between the four patient groups were evaluated by comparing group means with the Kruskal-Wallace procedure. Differences in RRs between high and low BCL-2- or BAX-expressing cases were evaluated by the Spearman ρ Correlation. Kaplan-Meier estimates of time to relapse or time to death (data censored as of August 1, 1997) were calculated within high- or low-expressing groups, and the groups were compared by the log-rank test. Differences in frequency of PCNA staining were evaluated by the t test.

RESULTS

Immunoblot Analysis of BAX Expression in Myeloma Cells. In initial studies, BM from 10 patients with active myeloma was obtained before initial treatment, and plasma cells were isolated by the CD38 immunoabsorption column (99% purity). Western blot analysis demonstrated a highly variable degree of BAX expression as well as BCL-2 expression in these 10 patients (Fig. 1A). Our BAX antiserum revealed a single M, 21,000 band typical of the most common form of BAX protein, p21-BAX-α. Additional studies demonstrated that BAX expression in myeloma cells was not altered by exposure to IL-6. This was demonstrated in five freshly obtained myeloma plasma cell samples and several myeloma cell lines. Samples from three of five patients are shown in Fig. 1B. CD38-column-purified plasma cells cultured with or without IL-6 (1000 units/ml) for 24, 48, or 72 h showed no alteration in BAX expression (although only the 0-h time point without IL-6 is shown, BAX expression did not change after 24–72 h in culture without IL-6). Although we have no data confirming that two of these primary myeloma cell populations can respond to IL-6 in other ways, patient 3’s myeloma cells increased their proliferation 2.1-fold after a 48-h exposure to IL-6 (1000 units/ml; not shown). Likewise, treatment of AF-10 (Fig. 1C, Line #1) and 8226 (Fig. 1C, Line #2) myeloma cell lines with IL-6 for 24–72 h showed no alteration in BAX expression, although the 8226 cell line (Fig. 1C, Line #2) responded to IL-6 with an induced protection against apoptosis (24), activation of the Jak-Stat signal pathway (25), and down-regulation of BCL-2 expression (Fig. 1C), and the AF-10 cell line (Fig. 1C, Line #1) responded by activation of the Erk pathway (26) and a proliferative response (27). In additional Western blot analyses not shown, IL-6 also had no effect on BAX expression in OCI-My5 and U266 myeloma cell lines. Thus, the variability in BAX expression seen in the first 10 myeloma cases (Fig. 1A) was not likely to be attributable to variable responsiveness to IL-6.

Relative Expression of BAX and BCL-2 in Malignant versus Nonmalignant Plasma Cells. We next examined archived BM tissue from 104 patients by IHC. The patients represented four separate clinical diagnostic groups: group I, reactive plasmacytosis (17 patients); group II, MGUS/smoldering myeloma (29 patients); group III, symptomatic myeloma at diagnosis (33 patients); and group IV, myeloma at relapse (25 patients). All of these patients were diagnosed and followed at the West Los Angeles Veterans Affairs Hospital, and their BM biopsies were identically fixed in formalin. The degree of plasma cell infiltration in these BMs (Table 1) is consistent with their individual diagnoses. The frequency and intensity of im-

Table 1  Degree of plasma cell infiltration of marrows of four patient groups

<table>
<thead>
<tr>
<th>Patient group</th>
<th>Percentage of plasma cell infiltration</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Reactive plasma cytosis (n = 17)</td>
<td>8.6</td>
</tr>
<tr>
<td>MGUS/smoldering myeloma (n = 29)</td>
<td>15.5</td>
</tr>
<tr>
<td>Myeloma at diagnosis (n = 33)</td>
<td>51</td>
</tr>
<tr>
<td>Myeloma at relapse (n = 25)</td>
<td>63</td>
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* Percentage of plasma cells in bone marrow biopsies of different patient groups. At least 300 cells were counted in differentials for each patient.
munistaining of plasma cells was blindly evaluated and recorded from 0 to 4+ as described in “Materials and Methods.” Mean scores for the four groups of patients are shown in Fig. 2 for BAX expression and, as a comparison, in Table 2 for BCL-2 expression. An increased BAX immunostaining in monoclonal plasma cells compared with polyclonal reactive plasmacytosis was detected (Fig. 2). The frequency and intensity of BAX staining were increased in the MGUS/smoldering myeloma group (group II) compared with reactive plasmacytosis (group I), and this was present at a borderline significance level (P = 0.082) for frequency (Fig. 2). BAX immunostaining frequency and intensity was significantly increased in the active myeloma group at diagnosis compared with reactive plasmacytosis (group I versus III; P = 0.001; Fig. 2) as well as in the active myeloma group at relapse (group I versus IV; P = 0.001; Ps not shown in Fig. 2). A further significant increase in immunostaining was detected in patients with active MM at diagnosis (group III) compared with the MGUS/smoldering myeloma group (group II; P = 0.003 for frequency and P = 0.005 for intensity; Fig. 2). The specificity of the antiserum for detection of human BAX protein in IHC was demonstrated by: (a) its ability to detect a single M₆ 21,000 protein band in immunoblot assays (Fig. 1); (b) its lack of reactivity with mouse BAX protein (6); and (c) its specifically inhibited reactivity when preabsorbed with human BAX peptide.

A similar trend showing increased BCL-2 immunostaining in monoclonal plasma cells compared with polyclonal reactive plasmacytosis was also detected (Table 2). As shown, a consistent increase in BCL-2 expression was observed (Table 2) in monoclonal plasma cells (groups II, III, or IV) compared with polyclonal reactive plasma cells (group I) when recorded as frequency or intensity of immunostaining. However, this difference was only significant (P < 0.05) when comparing group I to group III. Very little difference was found between patient groups II, III, and IV. Examples of BCL-2- and BAX-immunostained BMs are shown in Fig. 3.

Because complete “blinding” of myeloma BMs was impossible because a high percentage of plasma cell infiltration would strongly suggest the diagnosis of myeloma, we also separately analyzed the data obtained from the eight myeloma BMs (at diagnosis) with the smallest degree of plasma cell infiltration (mean, 24%; range, 15–29%). The BAX frequency and intensity scores of these eight cases were comparable with the entire group III (2.52 ± 0.42 and 1.72 ± 0.42, respectively) and were significantly higher (P < 0.05) than those of reactive and MGUS/smoldering myeloma BMs. Similarly, the BCL-2 frequency and intensity scores of these eight cases were comparable with the scores of the entire group III (3.47 ± 0.9 and 3.21 ± 0.83) and were significantly higher than scores obtained from reactive plasmacytosis BMs (P < 0.05). Because the plasma cell infiltration of these eight cases was very modest, these data support the objectivity of the above results.

We performed a cross-sectional comparison of group III (myeloma at diagnosis) to group IV (at relapse after therapy), assessing whether any therapy-induced adaptations in BCL-2 or BAX expression were apparent. BCL-2 expression remained similar (Table 2), but BAX expression modestly decreased in plasma cells of patients at relapse (Fig. 2). This decrease did not reach statistical significance.

We also attempted to correlate expression with clinical characteristics of the four major patient groups. Of 17 patients with reactive plasmacytosis (group I), 8 were diagnosed with AIDS, 4 had chronic infection, 4 had chronic liver disease, and 1 had an unspecified collagen vascular disease. No significant differences in BCL-2 or BAX expression were found between

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**Table 2** BCL-2 expression among four patient disease groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases</th>
<th>BCL-2 frequency</th>
<th>BCL-2 intensity</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>17</td>
<td>2.6471 ± 1.3082</td>
<td>2.2941 ± 0.9024</td>
</tr>
<tr>
<td>II</td>
<td>29</td>
<td>3.5862 ± 0.7080</td>
<td>2.8783 ± 1.0321</td>
</tr>
<tr>
<td>III</td>
<td>33</td>
<td>3.6667 ± 0.8165</td>
<td>3.1212 ± 1.1042</td>
</tr>
<tr>
<td>IV</td>
<td>25</td>
<td>3.4800 ± 0.8098</td>
<td>3.0200 ± 1.1132</td>
</tr>
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*Mean ± SD scores for BCL-2 frequency and intensity of plasma cell immunostaining.
Fig. 3 BMs stained for BCL-2 (A–C) or BAX (E–G) from patients with reactive plasmacytosis (A and E), MGUS (B and F), or myeloma at diagnosis (C and G). D and H are negative controls: myeloma at diagnosis where anti-BCL-2 or anti-BAX antibodies were preabsorbed with BCL-2 (D) or BAX peptide (H), respectively. Arrows, plasma cells in reactive and MGUS BMs. ×400.
these subgroups. Of the 29 patients in group II, 18 had MGUS and 11 had smoldering myeloma. Again, no significant differences in expression between these two groups were found. Of the 33 patients in group III, 28 were Durie-Salmon stage III and 5 were stage II, whereas all 25 patients in group IV were stage III. In these cohorts, no significant differences in expression were seen in stage II versus stage III patients, nor were there correlations between expression and β2-microglobulin levels, serum albumin, or hemoglobin levels (data not shown). Attempts to correlate BAX/BCL-2 expression with degree of plasma cell infiltration in BMs of each patient group were likewise unsuccessful.

Frequency of expression (percentage of immunopositive tumor cells) correlated well with intensity of expression for both proteins (not shown). However, no correlation between BCL-2 and BAX expression was observed in either frequency (percentage) or intensity of immunostaining (not shown).

**BAX/BCL-2 Expression versus Clinical Outcome.** For correlations with clinical outcome, only BMs from patients with active myeloma were evaluated. This cohort of patients consisted of 102 patients diagnosed between 1989 and 1996 at two hospitals, the West Los Angeles Veterans Affairs Hospital and Kaiser Permanente Hospital (Woodland Hills, CA). The Kaiser Hospital patients (n = 43) were all biopsied at diagnosis. All of these patients were stage III and were treated with M&P as induction therapy. The Veterans Affairs Hospital patients (n = 59) were either biopsied at diagnosis (n = 30) or on relapse (n = 29). All Veterans Affairs patients who were biopsied at initial diagnosis were treated with VAD as induction chemotherapy. Patients who were biopsied at relapse were then treated with second-line therapy consisting of either M&P, high-dose dexamethasone, or other agents used alone or in combination. Because the Kaiser and West Los Angeles Veterans Affairs patients were treated differently and their BMs were processed differently (different fixative), they were analyzed separately below.

**Response to Therapy.** Overall, in these 102 patients, there was no significant correlation between BAX expression and response to therapy. The lack of correlation was also true when the three patient groups were evaluated separately, and low-scored immunostaining patients (0–2+, 0–50% positively stained plasma cells) were compared with high-scored immunostaining patients (3–4+, 51–100% positively stained plasma cells). Of 43 Kaiser patients initially induced with M&P, the initial RR in those with low plasma cell BAX expression was 65%, and the RR in the high BAX expression group was 57%. Of 30 Veterans Affairs patients initially treated with VAD as induction chemotherapy, the RR in patients with low BAX expression was 58% and, in those with high expression, it was 67%. In 29 Veterans Affairs patients treated at relapse with varying regimens, the RR was 39% in low BAX-expressing groups versus 36% in high BAX-expressing groups. There were no significant differences in RR between low- versus high-expressing cases in any of these three groups, as determined by the Spearman ρ correlation. When the data were dichotomized differently by comparing scores of 0 and 1+ (as low-expressing cases) to 2, 3, and 4+ (as high-expressing cases), there were still no significant differences detected. Finally, comparisons of BAX expression assessed blindly by intensity of expression (0–2+ versus 3+, 4+, or 0–1+ versus 2–4+) rather than frequency of expression also demonstrated no significant differences.

The situation was similar for BCL-2 expression. There were no significant differences in RRs between low versus high BCL-2-expressing patient cohorts for the three patient groups. The RRs were 67% versus 60% [low (0–2+) versus high (3–4+)-expressing groups] in Kaiser patients initially induced with M&P, 50% versus 65% in Veterans Affairs patients induced with VAD, and 38% versus 38% in Veterans Affairs patients treated at relapse. As described above for BAX, when BCL-2 frequency of expression data were dichotomized differently (0–1+ versus 2–4+) or when expression was characterized by intensity of immunostaining, there were still no significant differences.

**Response Duration.** A significantly (P = 0.0073 by log-rank method) decreased remission duration was seen in Veterans Affairs patients studied at relapse (median duration of remission, 11 months) compared with either Veterans Affairs patients (median, 24 months) or Kaiser patients (median, 27 months) studied at diagnosis. This was anticipated because these results represent the second remission for Veterans Affairs patients studied at relapse, and these remissions tend to be shorter than initial remissions. However, BAX or BCL-2 expression had no bearing on response duration. This lack of correlation with immunostaining was true whether intensity or frequency of expression was assessed and in all three patient cohorts. Similarly, no significant differences were found when Cox regression analyses or log-rank tests were performed. The log-rank test was initially dichotomized between low expression scores of 0, 1, and 2 versus high scores of 3 and 4. We again attempted to dichotomize the data differently by comparing scores 0 and 1 (as low-expressing cases) to 2, 3, and 4 (as high-expressing cases), and still no significant differences were found.

**Survival.** The median survival of Kaiser Hospital patients, calculated from date of BM biopsy (which was the date of diagnosis) was 36 months. A similar median survival (32 months) was detected in Veterans Affairs Hospital patients likewise biopsied at diagnosis. As expected, the survival of Veterans Affairs Hospital patients, calculated from the date of BM biopsy upon clinical relapse, was lower (23 months). For all three of these patient cohorts, the level of BCL-2 expression, either assessed by frequency or intensity of immunostaining, had no bearing on survival. However, a cohort of patients with very low BAX expression (scores of 0–1+) demonstrated an unexpected prolonged survival. Of 43 Kaiser patients biopsied at diagnosis, 10 had BMs with 0–1+ BAX expression by frequency of immunostaining. The median survival of this group was 60 months, whereas the median survival of patients whose BAX frequency scores were 2–4+ was 28 months (Fig. 4, top; P = 0.0001 by log-rank method). A similar significant difference was detected in Veterans Affairs patients biopsied at diagnosis. Of these 30 patients, 9 had BMs with 0–1+ BAX frequency of expression. The median survival of these patients has not been reached, whereas the median survival of patients whose BAX frequency scores were 2–4+ was 20 months (Fig. 4, middle; P = 0.0003). Of 29 Veterans Affairs patients studied at the time of relapse, seven patients demonstrated low BAX frequency scores in their immunostained BMs. Their median
survival has not been reached, whereas patients with BMs demonstrating BAX frequency scores of 2–4 had a median survival of 15 months (Fig. 4, bottom; \( P = 0.0001 \)). A similar significant difference in survival was seen between low (0–1+) versus high (2–4+) BAX expression scores when immunostaining intensity was evaluated. This difference was significant with \( P = 0.0005, 0.008, \) and \( 0.0007 \) for Kaiser patients, Veterans Affairs at diagnosis patients, and Veterans Affairs at relapse patients, respectively (survival curves not shown). However, when survival of patients with BAX frequency scores of 2+ were compared with those with scores of 3–4+, no significant difference in survival was noted among any of the three cohorts of patients. Thus, a very low 0–1+ score specifically identified a subgroup of patients with long survival. When we compared the clinical characteristics of these low BAX-expressing cohorts (scores of 0–1+) to the remaining patients in their corresponding groups, there was no significant difference in serum \( \beta_2 \)-microglobulin, albumin, and hemoglobin levels or in degree of plasma cell marrow infiltration.

**Lack of Correlation between BAX and PCNA Expression.** A recent study (28) on the effects of BAX transfection in thymocytes and T lymphocytes supports a potential direct correlation between BAX expression and cell proliferation. Thus, the prolonged survival in low BAX-expressing cases could be attributable to an association with a particularly low proliferative potential in tumor cells. To investigate this possibility, we also stained our tissues for PCNA, used as a marker of tumor cell proliferation. Whereas Bouin-fixed tissues obtained from Kaiser Hospital patients demonstrated excessive nonspecific staining and could not be interpreted, formalin-fixed BMs, obtained from the Veterans Affairs Hospital, were successfully stained. In these latter specimens, PCNA-positive plasma cells were easily discernible from PCNA-negative plasma cells. Positive cells were diffusely stained in the nucleus, and plasma cell morphology was well maintained. Invariably, small numbers of early normoblasts or WBC precursors also stained positive for PCNA in all BMs and served as internal positive controls.

Plasma cells in BMs of patients with MGUS or smoldering myeloma were rarely positive for PCNA expression (mean, 1.3 ± 1%; Fig. 5). In contrast, the percentage of malignant plasma cells immunostained with PCNA antibody was 5.73 ± 3.5% (mean ± SD; median, 3%) in the MM at diagnosis group and 10.5 ± 4% (median, 11%) in MM cases studied upon relapse (Fig. 5). Both of these latter values are significantly \( (P < 0.05) \) higher than the values in the MGUS/smoldering MM group. In addition, the frequency of PCNA staining was significantly \( (P < 0.05) \) higher in the patients studied at relapse when compared with those studied at diagnosis.

We next compared the results of PCNA staining to BAX expression (Fig. 5). We separated the cases into those whose plasma cell BAX expression was 0–1+ versus 2–4+ because

![Fig. 4](image-url) Survival curves of Kaiser patients (top), Veterans Affairs patients biopsied at diagnosis (middle), and Veterans Affairs patients biopsied at relapse (bottom). ---, patients with BM BAX immunostaining frequency scores of 2–4+; ----, patients with scores of 0–1+.

![Fig. 5](image-url) Frequency of PCNA immunostaining of plasma cells in various patient groups.
the former were the patients who specifically had prolonged survival. Of patients whose BM was obtained at diagnosis, low BAX-expressing cases (score 0–1+) demonstrated a PCNA staining frequency of 6.33 ± 3.8% (mean ± SD; median, 3%) compared with a PCNA staining of 5.41 ± 3.5% (median, 4%) in high BAX-expressing cases. These values are not significantly different. For patients studied at relapse, there was a similar lack of correlation between BAX and PCNA expression; the percentage of PCNA staining of plasma cells in low BAX-expressing cases was 10.1 ± 3.9% (median, 11%) and 10.7 ± 3.5% (median, 11%) in high BAX-expressing cases.

**DISCUSSION**

Our results demonstrate that BAX expression is up-regulated in plasma cells of monoclonal gammopathies. The frequency and intensity of plasma cell immunostaining for BAX protein was significantly increased in patients with myeloma at diagnosis compared with those with reactive plasmacytosis. In addition, BAX expression was increased in plasma cells of MGUS/smoldering myeloma patients compared with reactive plasma cells. The $P$ for this difference in frequency of expression between reactive and MGUS/smoldering cases was only 0.082 when comparing all four group means with the Kruskal-Wallis procedure (Fig. 2) but was $<0.05$ when we only compared group 1 to group II (and excluded groups III and IV from the analysis). Furthermore, the differences in BAX expression between plasma cells of MGUS/smoldering myeloma patients versus those with myeloma at diagnosis were also significant ($P = 0.003$ and 0.0005 for frequency and intensity analyses, respectively).

The results in Table 2 also indicate that BCL-2 expression is increased in myeloma cells. This is consistent with a recent investigation by Miguel-Garcia et al. (29). The explanation for a malignancy-specific dysregulated BCL-2 expression in myeloma is unknown. The 14;18 chromosomal translocation is not malignancy-specific dysregulated BCL-2 expression in myeloma, occurring in only 10–15% of cases (32). One explanation is that BCL-2 gene amplification occurs during the course of tumor evolution with concomitant increases in BAX expression to retain a comparable BCL-2:BAX ratio within the plasma cells or vice versa. Another possible scenario is that BCL-2 expression is initially dysregulated, and because of the stabilizing effect of BCL-2 on BAX protein with resulting increased half-life (34), increased BAX expression ensues. However, the fact that BCL-2-transfected myeloma cells, ectopically expressing high levels of BCL-2, show no alteration in BAX expression (35) argues against this possibility. Finally, IL-6 exposure does not affect BAX expression in myeloma cell lines or freshly obtained specimens (Fig. 1), indicating that increased concentrations of this cytokine occurring during evolution of this malignancy is not likely an explanation for up-regulation of BAX.

No relationship was detected between either BAX or BCL-2 expression and RR to chemotherapy or duration of response. We also failed to detect significant correlations between BCL-2 expression and survival. A lack of correlation between high BCL-2 expression and decreased chemoresponsiveness and survival is consistent with previous studies, which evaluated smaller numbers of patients. In fact, Ong et al. (10) found higher frequency of BCL-2 plasma cell immunostaining in patients with long survival compared with those with shorter survival. Moreover, in another study, BCL-2 had no effect on responsiveness to M&P in 63 newly diagnosed patients (9), although BCL-2 expression in MM cells correlated with resistance to IFN.

In contrast to the results with BCL-2, however, BAX expression inversely correlated with survival. This was true for analyses of BAX intensity or percentage of BAX immunopositivity for all three patient cohorts. The effect on survival was not attributable to any influence on RRs or response duration. A similar relationship between BAX expression and cancer survival has been found in a limited number of studies in other tumor models (36–39). One possible explanation for this inverse correlation was that lower BAX expression in these subgroups of patients was simply a surrogate marker for lower BCL-2 expression, given the fact that BCL-2 alterations mirrored BAX alterations and that the BCL-2 assay might not have been able to distinguish significant differences in expression occurring in the high range of scores. We consider this explanation unlikely because a correlation between lower BCL-2 expression and prolonged survival should probably be associated with increased chemoresponsiveness and/or response duration (which it was not), given that low BCL-2 expression as a favorable indicator should be associated with increased apoptotic potential of treated tumor cells.

It is also possible that the prognostic importance of BAX expression is related to the corresponding BCL-2 expression. For example, in ovarian cancer patients (37), BAX expression is associated with poor outcome in general but is more important as a poor prognostic indicator when corresponding BCL-2 expression is high. In a similar study of patients with diffuse large cell lymphoma (39), low BAX expression was associated with lower survival when corresponding BCL-2 expression was low. However, in the patient cohort with high tumor cell BCL-2 expression, a situation similar to our patients with active myeloma, low BAX expression was significantly associated with enhanced survival, similar to what we saw.

Because BCL-2 overexpression inhibits proliferation (40) and an inverse relationship between BCL-2 expression and proliferative potential has been demonstrated in tumor cells in several different human tumor models (41, 42), we also consid-
erated the possibility that the reverse situation exists for BAX expression, i.e., a direct correlation between expression and tumor cell proliferation. A recent in vitro study (28) and clinical results in breast and ovarian cancer patients (36, 37) support this possibility. Thus, low BAX expression in myeloma cells might correlate with low tumor cell proliferation, therefore explaining the prolonged survival in this patient cohort. To test this hypothesis in our patients, we stained BM sections for PCNA as a marker for proliferation. Plasma cell PCNA positivity was very low in MGUS/smoldering myeloma BMs, significantly higher in active myeloma BMs, and higher in myeloma BMs studied at relapse compared with BMs studied at diagnosis. These results are consistent with prior studies (43, 44) that evaluated BM plasma cell proliferative status by immunostaining with Ki-67 antibody. However, we were unable to demonstrate a correlation between low BAX expression and low proliferative potential in this study. Thus, low BAX expression may correlate with another important survival parameter of the tumor-host interaction, which is unrelated to plasma cell proliferation.

In summary, our study clearly demonstrates an increase in BCL-2 and BAX expression in myeloma plasma cells when compared with reactive plasma cells. Plasma cells obtained from MGUS/smoldering myeloma patients demonstrate intermediate expression. BCL-2/BAX expression did not correlate with RR or response duration, but subsets of patients with very low BAX expression demonstrated long survival.

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Expression of BAX in Plasma Cell Dyscrasias

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