Moloney Murine Leukemia Virus-Induced Tumors Show Altered Levels of Proapoptotic and Antiapoptotic Proteins

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Moloney murine leukemia virus (M-MuLV) is a replication-competent, simple retrovirus that induces T-cell lymphomas when inoculated into neonatal mice. The tumor cells are typically derived from immature T cells. During preleukemic times, a marked decrease in thymic size is apparent in M-MuLV-inoculated mice. We previously demonstrated that this thymic regression is correlated with enhanced levels of thymocyte apoptosis (C. Bonzon and H. Fan, J. Virol. 73:2434-2441, 1999). In this study, we investigated the apoptotic state of M-MuLV-induced tumors. M-MuLV-induced tumors were screened for expression of the apoptotic proteins Fas and Bel-2 by three-color flow cytometric analysis. Single-positive (SP; CD4+ CD8− and CD4+ CD8+) tumor cells generally displayed lower cell surface expression of Fas than SP thymocytes from uninoculated control mice. Double-positive (DP; CD4+ CD8+) M-MuLV-induced tumor cells fell into two categories: those with normal high levels of Fas and those with low levels of Fas. Additionally, the vast majority of DP tumors showed elevated Bel-2 levels. The DP tumor cells retaining normal/high Fas expression were capable of transducing an apoptotic signal upon anti-Fas engagement. In addition, DP and CD4+ SP tumor populations displayed higher levels of Fas ligand than normal thymocytes with the same phenotypes. In contrast, CD8+ SP and CD4− CD8+ tumors did not show elevated Fas ligand expression. There was no significant correlation between Fas and Fas ligand expression in the DP tumors, suggesting that Fas Ligand expression was not the driving force behind Fas down-regulation. These results suggest that both the Fas death receptor and mitochondrial pathways of apoptotic death are active in M-MuLV-induced tumors and that they must be modulated to permit cell survival and tumor outgrowth.

Moloney murine leukemia virus (M-MuLV) is a replication-competent, simple retrovirus. When inoculated into newborn mice, it induces T lymphomas in 100% of the animals, with a mean latency of 3 to 4 months (9). Typically, tumor cells have phenotypes of developing thymocytes (CD4+ CD8+ [double positive (DP)], CD4+ CD8− [CD4+ single positive {SP}], CD4− CD8+ [CD8+ SP], or CD4− CD8− [double negative (DN)]). Due to its predictable pathogenic behavior, M-MuLV provides a system where changes that occur in mice before the onset of leukemia, as well as those that occur in end-stage tumors, can be examined.

M-MuLV leukemogenesis involves both early and late events. Early events, such as defects in bone marrow hematopoiesis, splenomegaly, and thymic atrophy, constitute a preleukemic state within the animal that is required for efficient disease induction (3, 8). Late events include long terminal repeat activation of proto-oncogenes and potential stimulation of growth factor receptors (6, 9, 10). Overexpression of proto-oncogenes presumably leads to uncontrolled cell proliferation and subsequent transformation. Although insertion events are essential for tumor formation, other changes must also presumably occur to allow for cell survival and tumor outgrowth. In particular, apoptotic pathways may be crippled in order to allow tumor cells to escape from cell suicide.

There are two general intracellular pathways for apoptosis (12). One involves the interaction of a death receptor with its ligand (i.e., Fas and Fas ligand), and the other involves the mitochondrial. The binding of Fas ligand to its receptor Fas causes the cytoplasmic domain of the receptor to associate with the adapter protein FADD (Fas-associated death domain). Through protein-protein interactions, FADD recruits procaspase-8 to a complex at the cell membrane, where it is cleaved into its mature form, caspase-8 (Flice). Caspase-8 cleaves downstream procaspases, such as caspase-3, ultimately resulting in the morphological and biochemical changes leading to programmed cell death (apoptosis).

The second apoptotic pathway involves the mitochondrion as a stress sensor in the cell. By an undetermined mechanism, mitochondria release cytochrome c from the intermembrane space into the cytoplasm upon receiving specific types of apoptotic stimuli (18). Once in the cytoplasm, cytochrome c associates with Apaf-1 and aids in recruitment of procaspase-9 to the complex. The subsequent activation of procaspase-9 results in cleavage of procaspase-3 to caspase-3 followed by the morphological and biochemical changes associated with apoptotic cell death. Bel-2 is a mitochondrial protein that can block the release of cytochrome c and prevent cell death by this pathway (16, 28).

We have previously demonstrated that M-MuLV-inoculated preleukemic mice display elevated levels of thymocyte apoptosis that correlate with disease pathogenicity, suggestive of a role in tumorigenesis. The focus of this study was to investigate how thymocytes in M-MuLV-inoculated mice ultimately overcome high preleukemic levels of thymocyte apoptosis and develop into tumors. Toward this end, the expression of apoptosis-associated proteins was studied in M-MuLV-induced thymic tumors. The results showed consistent alterations in apoptotic pathways.
TABLES AND METHODS

**Viruses and inoculation of mice.** Viral stocks were cell culture supernatants derived from NIH 3T3 cells productively infected with M-MuLV (7). Viral titers were determined by the UV/XC plaque assay (23). Neonatal NIH/Swiss mice were inoculated subcutaneously with 0.2 ml of wild-type M-MuLV stock (approximately 10^5 XC PFU). Moribund and control uninoculated adult mice were anesthetized by methoxyflurane inhalation and sacrificed by cervical dislocation. In infected NIH/Swiss mice, tumors generally present as thymic masses with or without nodal involvement. Thymi were dissected out, rinsed briefly in ice-cold phosphate-buffered saline (PBS), and immediately placed on ice. Thymic single-cell suspensions were prepared by gently teasing the organ apart in PBS and passing it through a 94-μm wire mesh (Belco Glass), allowing thymocytes to flow through, with stromal components remaining on the mesh. Thymocytes were then washed twice in ice-cold PBS and counted using a hemacytometer.

**Flow cytometric analyses for CD4, CD8, Fas, Fas ligand, and Bcl-2.** To determine cell surface Fas expression levels, approximately 10^6 cells were incubated with the antibody conjugates α-CD4-FITC (phycocerythrin: 1:80; Pharmingen), α-CD8-Cy (CyChrome: 1:40; Pharmingen), and α-Fas-FITC (fluorescein isothiocyanate: 1:200; Pharmingen) for 30 min on ice in the dark. In order to examine Fas ligand expression levels, approximately 10^6 cells were incubated with α-CD4-FITC (1:40) (Pharmingen), α-CD8-Cy (1:40), and α-Fas ligand-PE (1:40; Pharmingen) for 30 min on ice in the dark. In both cases, cells were then washed twice with ice-cold PBS and subjected to three-color flow cytometric analysis (FACSCalibur; Becton Dickinson). To investigate Bcl-2 expression levels, approximately 10^6 cells were first incubated with α-CD4-PE (1:80) and α-CD8-PE (1:80) (mouse Bcl-2-FITC set; Pharmingen) and resuspended in 0.0225% saponin in PBS-BSA, and either α-Bcl-2-FITC (1:10) or isotype control antibody (FITC conjugated; 1:10) (mouse Bcl-2 FITC set; Pharmingen) was added. Cells were then washed twice with ice-cold PBS-BSA, and subjected to flow cytometric analysis.

**RESULTS**

**Down-modulation of cell surface Fas expression on M-MuLV-induced tumor cells.** To investigate the levels of cell surface Fas expressed by M-MuLV-induced thymic tumors, the levels in normal thymocytes were first determined. Thymic single-cell suspensions from uninoculated control mice were incubated with α-CD4, α-CD8, and α-Fas and subjected to three-color flow cytometric analysis. Typical results for an uninoculated control mouse are shown in Fig. 1. The four individual CD4 CD8 populations (Fig. 1A) were analyzed for the presence of anti-Fas antibody Jo2 (10 μg/ml; Pharmingen). Cells were pelleted and incubated with annexin V-FITC and propidium iodide (TACS Annexin V-FITC apoptosis detection kit; R&D Systems) according to the manufacturer’s protocol and subjected to single-laser flow cytometry. Annexin V-high propidium iodide-low cells (early-stage apoptosis) and annexin V-high propidium iodide-high cells (late-stage apoptosis) were scored. The annexin V-high propidium iodide-high cells could also include necrotic cells in addition to late-stage apoptotic cells. However, based on forward and side angle scatter analysis, necrosis did not seem likely (i.e., unlike necrotic cells, the annexin V-high propidium iodide-high tumor cells appeared to be small). Tumor populations displaying apoptotic percentages below 1 standard deviation of the average apoptotic percentage from uninoculated control mice were categorized as displaying low levels of apoptosis, and those with percentages greater than 1 standard deviation of the mean percentage of uninoculated thymocytes were categorized as having elevated apoptosis. Those tumors with apoptotic percentages within 1 standard deviation of the mean percentage of uninoculated control thymocytes were classified as having normal apoptotic levels.

**Anti-Fas antibody engagement of Fas receptor and assessment of cell death.** Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin plus 2 μg of protein G per ml for 6.5 h in the absence or presence of anti-Fas antibody Jo2 (10 μg/ml; Pharmingen). Cells were pelleted and incubated with annexin V-FITC and propidium iodide (TACS Annexin V-FITC apoptosis detection kit; R&D Systems) according to the manufacturer’s protocol and subjected to single-laser flow cytometry. Annexin V-high propidium iodide-low cells (early-stage apoptosis) and annexin V-high propidium iodide-high cells (late-stage apoptosis) were scored. The annexin V-high propidium iodide-high cells could also include necrotic cells in addition to late-stage apoptotic cells. However, based on forward and side angle scatter analysis, necrosis did not seem likely (i.e., unlike necrotic cells, the annexin V-high propidium iodide-high tumor cells appeared to be small). Tumor populations displaying apoptotic percentages below 1 standard deviation of the average apoptotic percentage from uninoculated control mice were categorized as displaying low levels of apoptosis, and those with percentages greater than 1 standard deviation of the mean percentage of uninoculated thymocytes were categorized as having elevated apoptosis. Those tumors with apoptotic percentages within 1 standard deviation of the mean percentage of uninoculated control thymocytes were classified as having normal apoptotic levels.

**FIG. 1.** Cell surface Fas expression in uninoculated mouse thymocytes. Thymocytes from an uninoculated adult mouse were incubated with α-CD4-PE, α-CD8-Cy, and α-Fas-FITC and subjected to three-color flow cytometric analysis. (A) Distribution of the different CD4 CD8 populations. (B) GMFIs of four individual CD4 CD8 populations that were gated and assessed for cell surface Fas expression. In all cases, cell debris was gated out on the basis of forward and side angle scatter properties.
inoculation, times at which the normal thymus has already undergone complete physiological regression. Therefore, our analyses represented thymic tumor cells with negligible contributions from normal thymocytes. Figure 2 shows a typical analysis performed on thymic tumor cells from an M-MuLV-inoculated mouse. This tumor was comprised primarily of CD4⁺ SP cells. These tumor cells showed low levels of cell surface Fas expression (GMFI 21) compared to normal CD4⁺ SP thymocytes (Fig. 1B). Collectively, CD4⁺ SP tumor populations (Fig. 3A and B) displayed a statistically significant decrease in cell surface Fas expression in comparison to CD4⁺ SP cells from uninoculated mice (average GMFIs of 40 versus 114). CD8⁺ SP tumor populations also showed similar results (Fig. 3C and D) (GMFIs of 56 versus 97).

When DP tumor populations were analyzed for cell surface Fas (Fig. 4A), we also saw a significant decrease in Fas (average GMFI of 112, versus 147 for normal DP cells), although it was not as dramatic as seen in the CD4⁺ SP or CD8⁺ SP tumor populations. However, Fig. 4B shows a plot of the Fas levels expressed on normal DP cells from uninoculated mice compared to M-MuLV-induced tumors.

![Figure 2: Cell surface Fas expression in M-MuLV-induced CD4⁺ SP tumors.](image1)

![Figure 3: Cell surface Fas expression in CD4⁺ SP and CD8⁺ SP tumors.](image2)
pared to M-MuLV-induced DP thymic tumor populations. Tumor populations displaying GMFIs below 1 standard deviation of the value for normal thymocytes were categorized as displaying low Fas expression, while those displaying GMFIs within or greater than 1 standard deviation of the normal level were classified as having normal/high Fas expression. While many of the tumor populations (41 of 61) had lowered cell surface expression of Fas, a significant number (20 of 61) retained high to normal levels. In contrast, very few of the CD4\(^+\) SP and CD8\(^+\) SP tumors showed Fas levels in the normal range.

**Up-regulation of Bcl-2 in M-MuLV-induced DP tumors.** We also wanted to examine the levels of Bcl-2 expressed in M-MuLV-induced thymic tumors. Cells were stained with a-CD4 and a-CD8, and following a permeabilization step, a-Bcl-2, and subjected to three-color flow cytometric analysis. The different CD4 CD8 populations were scored for the percentage of cells expressing high levels of Bcl-2. As has been reported by other investigators (27), DP thymocytes generally expressed low levels of Bcl-2 (10\% expressed high levels of Bcl-2), whereas CD4\(^+\) SP, CD8\(^+\) SP, and DN thymocytes generally displayed higher percentages of cells expressing high levels of Bcl-2 (73, 32, and 38\%, respectively). For the purposes of our study, we concentrated on DP tumors since it would be more difficult to detect up-regulation of Bcl-2 in the other tumors, given the relatively high levels of Bcl-2 expression on the corresponding normal thymocytes.

Figure 5A and B show representative data for a DP tumor compared to DP cells from an un inoculated control animal. Figure 5C displays the percentage of DP cells expressing high levels of Bcl-2 for DP thymocytes from several un inoculated and DP tumor populations. Each bar represents the data derived from an individual animal. With a few exceptions, M-MuLV-induced DP tumors showed higher percentages of Bcl-2-high cells than normal DP thymocytes. On average (Fig. 5D), 11\% of DP thymocytes from un inoculated mice expressed high levels of Bcl-2, while on average 40\% of cells from M-MuLV-induced DP thymic tumors expressed high Bcl-2. Thus, up-regulation of Bcl-2 was a common feature of DP tumors.

**Fas signaling in M-MuLV-induced DP tumors.** As shown in Fig. 4B, we observed M-MuLV-induced DP tumors with low Fas and normal/high Fas levels, although both kinds of tumors showed high levels of Bcl-2 expression. We investigated the differences between the low Fas and normal/high Fas tumors, and why some down-regulated Fas while others did not. One possible explanation was that they represented tumors of DP thymocytes at different stages of differentiation or development. In particular, if in early DP thymocytes the Fas pathway of cell death is not active, then tumors derived from these cells would not be under selective pressure to down-regulate Fas, and cell surface Fas expression would remain normal/high. Conversely, tumors derived from later DP thymocytes might have to down-modulate an active Fas pathway in order to survive and escape death. To test this, we mimicked Fas ligand on M-MuLV-induced DP thymic tumor cells by addition of an anti-Fas antibody (Jo2) that is capable of engaging the Fas receptor and conveying a death signal (21). DP tumor cells were placed in culture for 6.5 h in the absence or presence of the Jo2 antibody, and the levels of apoptosis were assessed via annexin V and propidium iodide staining followed by flow cytometric analysis, as we have done previously (3).

Table 1 shows the results of this type of analysis on three tumors that expressed normal/high levels of cell surface Fas. (We focused on tumors comprised solely of DP cells so that annexin V-propidium iodide staining would measure apoptosis in these cells; in animals with multiple tumors of different phenotypes, annexin V-propidium iodide staining would measure apoptosis of all tumor cells combined.) Of the eight total un inoculated control thymi examined (two representative samples are shown in Table 1), none displayed increased apoptosis upon incubation alone, but all were very sensitive to Jo2 treatment (see also Table 2). All three tumors showed similar patterns; activation of apoptosis upon incubation in the absence of the Jo2 antibody (in comparison to thymocytes from uninoculated control mice that show slightly lower levels of apoptosis upon incubation alone) and further elevations in apoptosis upon incubation with Jo2. These results indicated that DP tumor cells retaining normal/high levels of cell surface Fas expression contained active and engagable Fas pathways, contrary to our hypothesis.

**Apoptotic levels in M-MuLV-induced tumors.** We were also interested in examining the overall levels of cell death occurring in M-MuLV-induced tumors. By annexin V-propidium iodide staining, we observed differences between the average initial levels of apoptosis (all CD4 CD8 populations measured together) in M-MuLV-induced tumors and uninoculated control thymi. Out of the 36 tumors analyzed in this fashion, 21 showed elevated apoptotic levels, while 9 showed lowered apoptotic levels and only 6 retained normal levels of apoptosis. This indicated that apoptotic pathways generally become activated in M-MuLV-induced tumors, leading to elevations in tumor cell death. In addition, as shown in Table 2, thymocytes from uninoculated control mice did not show increased apo-
ptosis upon incubation for 6.5 h unless they were treated with anti-Fas antibody (22% ± 5% versus 18% ± 3% and 85% ± 3%). Tumors initially displaying elevated levels of cell death could be further induced to apoptose by anti-Fas antibody to the maximal level for tumor cells (ca. 74%). Tumors displaying lowered levels of cell death at the time of sacrifice similarly showed sensitivity to anti-Fas antibody treatment. Interestingly, these tumors also showed elevated levels of apoptosis.

TABLE 1. Susceptibility of DP tumors with normal/high Fas to anti-Fas antibody

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fas expression (GMFI for DP cells)</th>
<th>% Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h b 6.5 h +Jo2 -Jo2</td>
<td></td>
</tr>
<tr>
<td>Uninoculated</td>
<td>160</td>
<td>19 ± 4 16 ± 5 87 ± 5</td>
</tr>
<tr>
<td>Tumor 1</td>
<td>149</td>
<td>23 ± 3 22 ± 3 82 ± 3</td>
</tr>
<tr>
<td>Tumor 2</td>
<td>116</td>
<td>29 ± 3 40 ± 3 86 ± 3</td>
</tr>
<tr>
<td>Tumor 3</td>
<td>141</td>
<td>9 ± 1 34 ± 1 90 ± 1</td>
</tr>
<tr>
<td>Tumor 4</td>
<td>182</td>
<td>28 ± 4 33 ± 4 92 ± 4</td>
</tr>
</tbody>
</table>

a Cells from three tumors comprised of solely DP cells displaying normal/high levels of Fas were placed in culture for 6.5 h in the absence or presence of anti-Fas antibody, as indicated. Apoptotic percentages were determined by staining with annexin V-propidium iodide.
b Time of sacrifice.

TABLE 2. Cell death levels in M-MuLV-induced tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>% Apoptosis (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h b 6.5 h +Jo2 -Jo2</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>22 ± 5c 18 ± 3d 85 ± 3d</td>
</tr>
<tr>
<td>With tumors</td>
<td></td>
</tr>
<tr>
<td>Low death (9)</td>
<td>12 ± 3 41 ± 3 74 ± 3</td>
</tr>
<tr>
<td>Normal death (6)</td>
<td>22 ± 3 52 ± 3 71 ± 3</td>
</tr>
<tr>
<td>Elevated death (21)</td>
<td>48 ± 15 54 ± 14 73 ± 15</td>
</tr>
</tbody>
</table>

a Cells from thymi of uninoculated control mice or from 36 M-MuLV-induced tumors were cultured as described for Table 1 and analyzed by annexin V-propidium iodide staining for the levels of cell death. Individual CD4 CD8 populations within a given tumor were not distinguished in this analysis.
b Time of sacrifice.
c n = 8.
d n = 9.
e M-MuLV-induced tumors were classified based on whether they showed low, normal, or elevated levels of apoptosis at the time of sacrifice. The numbers of tumors (of a total of 36) in the categories are given in parentheses.
after incubation, even in the absence of anti-Fas antibody, suggesting that the apoptotic machinery in the low death tumors is in a primed state that can be activated upon removal from the animal and incubation in culture. Tumors displaying normal apoptotic levels at the time of sacrifice also displayed reduced Fas ligand expression with high Fas down-modulation in any of the M-MuLV-induced tumor types.

Up-regulation of Fas ligand expression by some M-MuLV-induced thymic tumors. Another possible explanation for the reduced Fas expression that we explored was expression of Fas ligand by the tumor cells themselves. Simultaneous expression of Fas and Fas ligand would provide tumor cells with a potent proapoptotic stimulus and a selective force for down-regulation of Fas. While this was possible for the CD4+ SP and DP tumors that frequently expressed elevated levels of Fas ligand, it was not the mechanism for CD8+ SP tumors that generally did not show increased Fas ligand expression. However, while essentially all CD4+ SP tumors showed very low Fas levels, some, but not all, showed increased Fas ligand. Therefore, it was unlikely that Fas ligand induction was the mechanism for down-modulation of Fas in these tumors. Moreover, in DP tumors, there was no relationship between levels of cell surface Fas and Fas ligand, as shown in Fig. 6B. Thus, there was no evidence for induction of Fas ligand in these tumor cells as the driving force for down-modulation of Fas in any of the M-MuLV-induced tumor types.

It is still possible that Fas ligand might be the selective force for the Fas down-modulation, but that Fas ligand is produced by the stromal compartment of the thymus and not the thymocytes. In the normal thymus, Fas ligand is predominantly expressed by stromal cells within either the cortical or medullary regions (11). Moreover, we previously showed that thymocytes from preleukemic M-MuLV-infected mice show enhanced levels of apoptosis (3). It will be interesting to examine stroma from preleukemic mice for expression of Fas ligand.

Studies performed by other groups have suggested a strategy
by which MuLV-infected cells use the Fas-Fas ligand interaction to evade immune T-cell recognition (22). Uninfected C57BL/6 mice (H-2b) generate an anti-MuLV CTL response against AKR MuLV-infected tumor cells, whereas AKR.H-2b mice (congenic at the H-2 locus) generally do not. (AKR.H-2b mice are infected with endogenous AKR MuLV that activates at birth.) In vitro killing of AKR MuLV-infected tumor cells by C57BL/6 antiviral CTLs can be inhibited by addition of AKR.H-2b spleen cells (virus infected). The inhibitory cells have been termed veto cells. Experiments with mouse lines genetically deficient in Fas or Fas ligand indicated that the veto cells function by expressing Fas ligand and triggering Fas-mediated activation-induced cell death of the virus-specific CTLs. Similarly, the M-MuLV-induced DP and CD4 SP tumors studied here might also have veto activity against any virus-specific CTLs, since they often expressed high levels of Fas ligand.

We also found that approximately one-third of M-MuLV-induced DP tumor populations did not show down-regulation of cell surface Fas. In the normal thymus, DP cells are extremely susceptible to apoptotic cell death, since thymocytes at this stage of development undergo positive and negative selection. As the apoptotic pathways associated with positive and negative selection remain to be conclusively elucidated, it is possible that apoptotic pathways may be differentially primed in thymocytes undergoing these selection processes. Accordingly, we hypothesized that tumors retaining normal/high levels of cell surface Fas originated from thymocytes in which the Fas pathway was not primed or active. However, DP M-MuLV-induced tumors that retained normal/high Fas expression could be induced into apoptosis by engaging Fas with Jo2 antibody, suggesting that the Fas pathway was indeed functional in these tumors. Thus, this hypothesis was not verified.

The overall apoptotic rate or level of different tumor populations was also of interest. It was important to determine if tumor populations with down-modulation of apoptotic pathways show elevated or modest levels of apoptosis. However, this has been technically challenging, since M-MuLV-induced thymic tumors frequently contain tumor cells of more than one CD4 CD8 phenotype. To some extent, we have addressed this by focusing on tumors that contained only one CD4 CD8 phenotype for certain analyses (e.g., Table 1) and also looking in general (without CD4 CD8 discrimination [Table 2]). The isolation of individual CD4 CD8 tumor populations from M-MuLV-induced tumors and un inoculated control mice is currently under way.

In M-MuLV-induced DP tumor populations, the antiapoptotic protein Bcl-2 also was elevated. This suggested that the mitochondrial pathway of apoptotic death was active in these cells and that down-modulation via increases in Bcl-2 was important for tumor cell survival. Alternatively, as the Bcl protein provides cross-talk between the death receptor and mitochondrial pathways of apoptosis, increased Bcl-2 levels could also potentially counter apoptotic signaling originating from death receptor stimulation (4, 17, 19). The finding of elevated Bcl-2 in DP tumors was not surprising, as it is very common to find elevated levels of Bcl-2 in malignancies (human follicular B-cell lymphoma, acute myeloid leukemia, hormone-independent adenocarcinomas of the prostate, colorectal adenocarcinoma, small cell and non-small cell lung carcinoma, adenocarcinoma of the breast, neuroblastomas, retinoblastomas, etc.) (1, 2, 5, 13, 15, 20). It will be interesting to examine the expression of other Bcl-2 family members in M-MuLV-induced thymic tumors, most notably those that have proapoptotic effects (e.g., Bax). This is important because Bcl-2 family proteins physically interact with one another, and these interactions can affect apoptosis negatively or positively. At the time these experiments were performed, reagents to efficiently detect other murine Bcl-2 family members in a similar fashion were not readily available.

In the future, it will be interesting to investigate the molecular mechanisms of the modulations in pro- and antiapoptotic proteins in M-MuLV-induced tumors. Although the general mechanisms of the expression of these proteins remain largely unknown, possibilities include transcriptional modulation (e.g., p53 repression of bcl-2 gene expression) and, in the case of Fas and Fas ligand, transport to the cell surface.

In summary, alteration of apoptotic pathways relative to normal thymocytes of the same phenotype was a general property of M-MuLV-induced thymic tumors. The alterations affected the death receptor pathway (down-modulation of Fas) and (where measurable, in DP tumors) the mitochondrial pathway (up-regulation of Bcl-2). While we were not able to identify the mechanisms for this down-regulation, some possibilities were eliminated. What might be the driving forces behind these changes? In order for cells in a tumor to increase in number, cell division must outpace apoptosis; apoptotic rate is determined in turn by the balance between proapoptotic and antiapoptotic signals. At the same time, the induction of apoptotic pathways has been described for numerous tumor types (14, 24, 26), and the observed activation of apoptosis in M-MuLV-induced tumors may result from similar mechanisms. In order for M-MuLV-induced tumors to grow, reductions in proapoptotic signals and increases in antiapoptotic signals may be necessary to counteract the increased apoptosis.

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