Bax Accelerates Tumorigenesis in p53-deficient Mice

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ABSTRACT

Bax is a Bcl-2 family member that promotes apoptosis and counters the protective effect of Bcl-2. Bax is a downstream effector of p53-induced apoptosis and is transcriptionally regulated by p53. Moreover, the introduction of Bax deficiency accelerates the onset of tumors in transgenic mice expressing truncated large T antigen. These results implicate Bax as a tumor suppressor. Consequently, we asked whether the levels of Bax expression would influence tumor development by comparing Bax-deficient and Bax transgenic mice in the presence or absence of p53. We found that Bax-deficient mice did not display an increased incidence of spontaneous cancers when followed for >1.5 years. In addition, Bax-deficiency did not further accelerate oncogenesis in mice also deficient in p53. We generated Lckpr-Bax transgenic mice to examine the effects of overexpressed BAX on T-cell development and tumorigenesis. Lckpr-Bax mice show increased apoptosis consistent with the pro-apoptotic function of Bax. The introduction of p53-deficiency did not interfere with BAX-induced apoptosis; this is consistent with BAX operating downstream or independent of p53. However, we found that Lckpr-Bax/p53-deficient mice have an increased incidence of T-cell lymphomas when compared with p53-deficient mice. The Lckpr-Bax transgenic mice have an increased percentage of cells in cycle. These findings extend previous work suggesting that Bcl-2 family proteins regulate proliferation as well as cell death. We conclude that BAX-induced proliferation is synergistic with a defect in apoptosis contributed by p53-deficiency. Thus, the dual roles of BAX can either accelerate or inhibit tumorigenesis depending on the genetic context.

INTRODUCTION

The Bcl-2 family contains a number of related genes that are critical regulators of apoptosis (1). The family can be divided into those that inhibit (Bcl-2) and those that promote (Bax) cell death. Inhibition of apoptosis by Bcl-2 promotes oncogenesis as illustrated by the presence of the t(14;18) translocation in follicular B-cell lymphoma (2–4) and the ability of Bcl-2 to promote tumors in transgenic mice (5). Furthermore, a number of findings implicate the loss of function of the pro-apoptotic family member Bax in oncogenesis. First, Bax can be a direct transcriptional target for p53 (6). p53 is the most frequently mutated gene in human cancer, and its loss is associated with both deregulated proliferation and resistance to apoptosis (7). Animal studies also support Bax as an effector of p53-dependent apoptosis. Bax deficiency decreases apoptosis and accelerates oncogenesis in truncated SV40 Tag transgenic mice susceptible to brain tumors (8). Also, haplo-insufficiency of Bax accelerates oncogenesis in Tag transgenic mice susceptible to mammary tumors (9). In vitro studies of E1A-induced transformation also show that Bax deficiency decreases apoptosis and promotes transformation in this p53-dependent in vitro model (10). Together these findings provide evidence that Bax partly mediates the p53-dependent apoptosis induced by these two potent viral oncoprogens.

Studies of human malignancies have also implicated BAX as an important tumor suppressor. For example, in colorectal cancer (11), gastric carcinoma (12), and acute lymphoblastic leukemia, frameshift mutations in BAX are found frequently in tumors with the microsatellite mutator phenotype. Mutations in BAX have been described in a number of human hematopoietic tumor cell lines (13) as well as directly from gastrointestinal cancer (14). Retrospective studies of human malignancies have examined the relationship between BAX expression by immunohistochemistry or immunoblotting and clinical outcome. Reduced expression of BAX is associated with poor clinical outcome in ovarian cancer (15), metastatic breast adenocarcinoma (16), and squamous cell carcinoma (17). In contrast, increased BAX expression correlated with a high rate of relapse in childhood acute lymphoblastic leukemia (18).

Thus, a number of lines of evidence in both mice and humans suggest BAX is a tumor suppressor because of its ability to promote apoptosis. However, these studies have limitations. To date, studies done in mice have used potent viral oncogens to induce oncogenesis. The relevance of these models to spontaneously or naturally occurring tumors is unknown. Similarly, the studies in humans are correlative, retrospective studies that do not prospectively establish a causative role. In this study we asked whether the levels of Bax expression could directly influence tumor development in mice in the absence of viral oncogenes. We find that Bax-deficiency alone or in combination with p53-deficiency do not predispose to malignancy. In contrast, we also find that overexpression of BAX accelerates oncogenesis in a p53-deficient background. Before malignant transformation, BAX overexpression increases apoptosis while it results in an increased percentage of cycling cells. These findings extend previous observations that Bcl-2 family members regulate both apoptosis and cell division and indicate that the interplay of these activities affects malignant transformation.

MATERIALS AND METHODS

Materials and Mice. Bax-deficient mice were genotyped by PCR as described previously (19, 20). p53-deficient mice obtained from the Jackson Laboratory (Bar Harbor, ME) were genotyped by PCR as described previously (21, 22). When possible, p53-deficient mice were used for mating to minimize the number of animals. Thus, littermate controls included heterozygote animals. Bcl-2-deficient mice were genotyped by PCR as described previously (19). The Mouse Genome Project (23) was used to establish the Lckpr-Bcl-2 transgenic mice were genotyped by PCR as described previously (19). The Lckpr-Bcl-2 construct was generated by blunt-end ligation of the full-length murine cDNA sequence of Bax (23) into the BamHI site of the Lckpr-Bcl-2 transgenic mice (24). Pronuclei from fertilized oocytes (C57BL/6-C3H/He Fl crosses) were injected with linearized DNA and the oocytes incubated overnight before transplantation into the oviduct of pseudopregnant females. Seven independent lines were generated and the three lines showing the highest level of expression were chosen for subsequent analysis. Mice were back-crossed and maintained on the C57BL/6 background.

For developmental studies, Lckpr-Bax line 38 transgenic males were mated overnight to C57BL/6 females. The day of plug identification is defined as day 0.5. At the indicated time, the pregnant females were sacrificed and the embryos dissected. Each of the thymi were removed with the aid of a dissecting microscope and examined to ensure that both lobes could be identified.

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4 The abbreviations used are: TAg, large T antigen; PI, propidium iodide; BrdUrd, bromodeoxyuridine.

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Thymi were placed in individual wells of a 24-well plate. Thymocytes were isolated by first shearing the gland with two needles and then passing the cells through a 22-gauge needle multiple times. The cells were centrifuged, and in some experiments, samples were treated with hypotonic lysis buffer [0.83% NH₄Cl and 10 mM Tris (pH 7.2)] for 5 min. As the RBC lysis reduced the cell yield, all sample embryos from a given litter were treated identically and then normalized to the average for the control animals in the litter. The tails from the embryos were used for PCR genotyping.

For reasons which are not clear, the LcK⁺/Bax line 1 demonstrated a phenotype that varied with the background strain (data not shown). The differences in thymus size and percentage of cycling cells was much more pronounced on the C3H/He background when compared with the C57BL/6 background. These differences were not observed for the other transgenic lines. The LcK⁺/Bax mice were genotyped using PCR with a forward Bax cDNA primer (5'-GAGCTGAATGACACCATCATG-3') and a reverse human growth hormone primer (5'-GTAGCCATGCGCATGCTG-3'). The primers were used at 0.4 μM and produced a 500-bp product (Lines 1 and 8) or a 350-bp product (Line 38) after 30 cycles (94°C × 1 min; 60°C × 1 min; and 72°C × 1 min). Bcl-2 exon III cDNA primers (0.2 μM) that amplify a 250-bp product were included in each tube to serve as a positive control for each reaction (5'-CTTTTGGGAACTGTAGGCAGC CCG' and reverse 5'-ACAGCGCTGGCTTTCATGATAC').

Aging Studies. Mice of the appropriate genotypes were mated for two or more generations to obtain animals for the aging study. Upon entry into the aging study, animals were examined weekly for signs of illness or malignancy. Sick animals were monitored more frequently and euthanized when necessary to prevent suffering. Bax-deficient mice were monitored for a minimum of 1.5 years. Animals that survived the duration of the study were killed, and a necropsy was performed. Because these animals did not show overt signs of disease, tumors found in these mice were censored from the analysis because it is not known whether they would have died or become ill from the tumor. Despite these efforts, the majority of Bax-deficient mice that died while on study were not noted to be sick before their death. When possible, necropsies were performed on dead animals to determine whether the animals had gross evidence of tumors. Tumors were then confirmed by fixation with formalin and histological examination after H&E staining. A hematopathologist examined the sections and confirmed the tumor type. All mice were maintained in the animal facility at the University of Iowa (Iowa City, IA). Statistical analysis was performed with the StatView Program (SAS Institute Inc.) using Kaplan-Meier cumulative survival and the log-rank (Mantel-Cox) test to determine whether differences in survival were significant. Analysis was performed by comparing Bax-deficient mice, Bax+/+, Bax−/−, and both together. p53-deficient mice with and without the Bax or Bcl-2 transgene were compared with each other.

Cell Preparation and Analysis. Single-cell suspensions were prepared from the thymi by dispersing the organs between two glass slides in isotonc saline. When necessary, RBCs were removed by a 5-min incubation in hypotonic lysis buffer [0.83% NH₄Cl and 10 mM Tris (pH 7.2)], and the viable cell counts were determined using a hemocytometer and trypan blue exclusion (25). Cells were cultured in RPMI 1640 supplemented with 10% FCS, Pen-Strep, glutamine, and 2-mercaptoethanol (100 μM). Thymocyte viability was determined using double staining with Annexin V-FITC (Trevigen) and PI as instructed by the manufacturer. Viability after irradiation was normalized to the untreated control samples by the following equation: % viable with irradiation% viable without irradiation at the same time point. Cell cycle analysis was performed by analyzing PI-stained nuclei on a flow cytometer equipped for doublet discrimination (FACScan or FACS Calibur from Becton Dickinson). Briefly, −1 × 10⁶ cells were pelleted and resuspended in 0.5 ml of Krishan reagent before analysis (26). The percentage of cycling cells (SSC/M1) was determined by examining histograms after gating-out doublet events on the basis of FL2-2A versus FL2-2W. CellQuest software (Becton Dickinson) was used for both the acquisition and analysis.

BrdUrd (Sigma) uptake in thymocytes was determined after i.p. injection of BrdUrd (5 mg/mouse). Thymocytes were harvested 1 h after injection and a single-cell suspension was prepared as above. BrdUrd staining was performed using the PermaCyte-FP kit following the manufacture directions (BioErgonomics, St. Paul, MN). Briefly, thymocytes were washed in PBS, centrifuged (1000 × g), and resuspended in 70% ethanol for fixation (−20°C for 30 min). After centrifugation, the cells were resuspended in 4 N HCl and incubated at room temp for 30 min to denature the DNA and expose the BrdUrd. After treatment with permeabilization buffer, cells were stained with anti-BrdUrd FITC (Becton Dickinson) by incubation for 30 min at room temperature. After washing with permeabilization buffer, the cells were resuspended in PBS containing 20 μg/ml PI. Mice that were not injected with BrdUrd were used as negative controls to set the level of background staining. Acquisition and analysis was performed on the FACS Calibur (Becton Dickinson) using doublet discrimination (FL2 area versus width).

RESULTS

Tumor Development in Bax-deficient Mice Bax-deficient mice show lineage-specific aberrations in cell death with increased numbers of germ cells, lymphocytes, and neurons in young or mature mice (20, 27, 28). To determine whether this lineage-specific hyperplasia is associated with increased malignancy, a cohort of Bax-deficient mice and control littermates were monitored for signs of tumor development for 1.5–2 years. Although the Bax-deficient mice showed decreased overall long-term survival (Fig. 1A), the decreased survival could not be attributed to neoplasia because the tumor-free survival was no different between groups (Fig. 1B). Necropsies performed on the mice, either at the end of the study or at the time of their deaths, did not identify an obvious cause of death in the majority of Bax-deficient mice (Table 1).

![Fig. 1. Aging study of Bax-deficient mice. The overall survival (left) and tumor-free survival (right) of Bax-deficient (−/−, ○), heterozygous (+/−, ●), and wild-type (+/+; △) mice is shown. All animals were monitored weekly for signs of illness or tumor development. Necropsies were performed on all mice (when possible) to determine whether the animals showed evidence of tumor development. Tumor-free survival of Bax-deficient mice was not significantly different from that of wild-type or heterozygous mice. In addition, heterozygous and wild-type mice were not significantly different from each other. For disease-free survival, Bax-deficient mice showed reduced survival relative to heterozygous alone (P = 0.0263) and heterozygous or wild type (P = 0.0353). However, disease-free survival compared with wild type alone did not achieve significance in this cohort (P = 0.0644). P values were derived using the log-rank (Mantel-Cox) test and the StatView statistical package as described in "Materials and Methods.”](image-url)
Bax has been shown to mediate apoptosis in pathways that are independent of p53 (28). Furthermore, murine Bax and human BAX may be regulated differently in regards to the role of p53 (29). Thus, to determine whether Bax and p53-deficiencies would cooperate in tumor development, Bax-deficient mice were mated to p53-deficient mice. Bax/p53-doubly deficient mice were monitored for tumor development and compared with littermate control mice. Doubly deficient mice did not develop tumors at a significantly different rate than littermates singly deficient for p53 (Fig. 2A). One explanation for this result is that p53-deficiency results in a substantial apoptosis defect where Bax-deficiency provides no additional resistance to death, consistent with Bax being downstream of p53. As an additional test of this hypothesis, the ability of Bcl-2 overexpression to promote tumor development in p53-deficient mice was determined. Lck-nor-Bcl-2 transgenic mice were chosen for this study because the overexpression of Bcl-2 in developing and mature T cells results in dramatic resistance to a number of apoptotic stimuli (24). Furthermore, these mice progress to T-cell lymphomas, albeit with a long latency (5). Because p53-deficient mice are also prone to the development of T-cell lymphomas (22), the Lck-nor-Bcl-2/p53-deficient mice were generated and monitored for tumor development. In contrast with our findings with Bax-deficiency, Bcl-2 was able to dramatically accelerate oncogenesis in p53-deficient mice (Fig. 2B). Because Bcl-2 expression is restricted to T cells in these transgenic mice, the incidence of lymphoma-free survival is most pertinent. In this case, seven of seven Lck-nor-Bcl-2/p53-deficient mice developed thymic lymphomas, whereas only four of seven of the nontransgenic p53-deficient littermates developed thymic lymphomas. When a Kaplan-Meier lymphoma-free survival curve was analyzed using the log-rank test, the P = 0.006 (data not shown). These results clearly show that Bcl-2 can promote tumor development in p53-deficient mice. This finding is consistent with experimental evidence that Bcl-2 can block p53 independent as well as dependent deaths. This also illustrates that overexpression of an anti-apoptotic member, Bcl-2, would have a broader effect than loss of expression of a single pro-apoptotic member, BAX.

**Generation and Characterization of Lck-nor-Bax Transgenic Mice.** Because Bax has been implicated as a downstream effector of p53 apoptosis, we next asked whether overexpression of Bax would alter tumor development in p53-deficient mice. Two previous studies have shown that a high percentage of p53-deficient mice develop thymic lymphomas. Jacks et al. (22) found that 44 of 56 (71%) of

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**Table 1 Summary of tumor watch for Bax-deficient mice**

<table>
<thead>
<tr>
<th>Outcome group</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. entered</td>
<td>29</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>No. completed study</td>
<td>16</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>No. necropsy-confirmed tumor</td>
<td>6</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>No. necropsy-inconclusive</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>No. non-tumor cause of death</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>No. censored</td>
<td>5</td>
<td>4</td>
<td>12</td>
</tr>
</tbody>
</table>

* Mice that completed the study are those which survived a minimum of 1.5 years without signs of illness or tumors.
* Includes two mice with glomerulonephritis, one with an abscess, and one with extramedullary hematopoesis (spleen).

These mice were censored from the tumor-free survival analysis because adequate necropsy could not be performed because of either a long delay in identification or cagemate cannibalism. Also includes one --/ mouse with a protracted rectum.

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Fig. 2. Lck-nor-Bcl-2, but not Bax-deficiency, accelerates tumorogenesis in p53 --/ mice. A, tumor-free survival of Bax/p53 double-deficient mice (+) and p53-deficient/Bax +/+ or +/− (△) littermates is plotted. B, tumor-free survival of Lck-nor-Bcl-2/ p53-deficient (●) and p53-deficient mice alone (△) are shown. Tumor surveillance and statistical analysis were performed as described in Fig. 1 and in “Materials and Methods.” When lymphoma-free survival was examined for the Lck-nor-Bcl-2 transgenic mice, the difference was even greater, with P = 0.006 (data not shown).

**Fig. 3. Increased apoptosis and lymphopenia in Lck-nor-Bax transgenic mice.** A, mice 4–6 weeks of age from three independently derived Lck-nor-Bax transgenic lines (Line 1, Line 38, and Line 8) and littermate controls (Neg) were sacrificed and the thymi dissected. The total number of viable thymocytes was determined by trypan blue staining. The percent of thymocytes from each animal relative to the mean of the nontransgenic littermates is shown. The data show that the number of cells isolated from transgenic mice was consistently lower than from the nontransgenic littermates. B, thymocytes from 4-week-old Lck-nor-Bax (Line 38) and littermate control (Neg) mice were isolated and placed in culture in the presence of 10% FCS. Viability was determined at the indicated times by Annexin-V/PI exclusion as indicated in “Materials and Methods.” Mean/SD of triplicate samples is shown. The experiment is representative of more than three experiments.
p53-deficient mice developed lymphoma, whereas Donehower et al. (30) described very similar results in independently derived mice (20 of 26; 77%). To examine whether Bax could suppress oncogenesis, transgenic mice overexpressing Bax in T cells were generated using the Lck proximal promoter. Thymocyte counts from seven independently derived lines of transgenic mice were determined. Of seven lines generated, we found three lines (lines 1, 8, and 38) that demonstrate significant lymphopenia (Fig. 3A). Two of these lines (lines 1 and 38) were used for additional studies. A number of experiments indicate the lymphopenia is secondary to increased apoptosis. First, in vitro survival of isolated thymocytes is decreased in cells overexpressing Bax (Fig. 3B). In addition, freshly isolated thymocytes showed increased evidence of apoptosis based on both terminal deoxynucleotidyltransferase-labeling and quantitation of hypodiploid DNA (data not shown). In vivo, the thymus showed moderately increased terminal deoxynucleotidyl transferase-mediated nick end labeling. Freshly isolated thymocytes from transgenic mice have an increased percentage of cycling cells, thereby making decreased generation unlikely (Fig. 6A). Overall, these data demonstrate that overexpression of Bax increases the susceptibility of thymocytes to cell death.

**Tumor Susceptibility of Lck\(^{pr}\)-Bax/p53-deficient Transgenic Mice.** The Lck\(^{pr}\)-Bax transgenic mice were next crossed to p53-deficient mice. Lck\(^{pr}\)-Bax/p53-deficient mice and the p53-deficient nontransgenic littermates were monitored weekly for evidence of tumors. Surprisingly, Lck\(^{pr}\)-Bax line 38 decreased the tumor-free survival of p53-deficient mice (Fig. 4A). Assessment of lymphoma-free survival was also significantly decreased in Lck\(^{pr}\)-Bax/p53-deficient mice (Fig. 4B). Nearly identical results were found when tumor-free survival was assessed in Lck\(^{pr}\)-Bax line 1/p53-deficient mice (Fig. 4C). Similar results for two independent transgenic lines demonstrate the results are not dependent on the integration site of the transgene.

**Effects of Lck\(^{pr}\)-Bax on Apoptosis in p53-deficient Mice.** Several recent reports suggest that Bax may demonstrate paradoxical effects on apoptosis (31, 32). In addition, Bax mediated apoptosis is attenuated in some p53-deficient cells (10). Thus the ability of Bax to promote apoptosis of thymocytes in p53-deficient mice was examined. The presence of the Lck\(^{pr}\)-Bax transgene was found to promote in vitro apoptosis of thymocytes independent of p53 expression (Fig. 5A). In vivo, the lymphopenia of Lck\(^{pr}\)-Bax transgenic mice was also independent of p53 (data not shown). Of note, overexpression of Bax proved insufficient to confer sensitivity of thymocytes to \(\gamma\)-irradiation in p53-deficient mice (Fig. 5B). These findings and previous results (20) demonstrate that p53 and Bax are independent regulators of cell death in thymocytes.

**Altered Proliferation of Lck\(^{pr}\)-Bax Transgenic Thymocytes.** Freshly isolated thymocytes from Lck\(^{pr}\)-Bax transgenic mice were found to have markedly increased percentages of cells in the S/G2-M phase of the cell cycle, based on PI staining. (Fig. 6A). Because the animals are lymphopenic, one possibility is that the increased proliferation serves as a compensatory adjustment to altered homeostasis. However, previous studies of Bax transgenic mice using the CD2 promoter noted increased proliferation in the presence of normal numbers of thymocytes (33, 34). This suggests the proliferative defect is not secondary to lymphopenia. If the increased percentage of
cycling cells is secondary to lymphopenia, we reasoned the lymphopenia would precede changes in proliferation. The chronology of defects in proliferation and thymocyte number was determined at various developmental ages. The thymocyte number and proliferative index of Lck<sup>pr</sup>-Bax transgenic embryos at E16.5 were not significantly different from nontransgenic littermates (Fig. 6B). Over the next two days of embryonic development, the Lck<sup>pr</sup>-Bax transgenic mice synchronously displayed alterations in cell cycle and cell number (Fig. 6, B and C). We conclude that Bax overexpression alters both apoptosis and proliferation of developing T cells.

**BrdUrd Uptake Is Increased in Lck<sup>pr</sup>-Bax Thymocytes and Is Independent of p53 Status.** To further characterize the cell cycle abnormalities in Lck<sup>pr</sup>-Bax transgenic mice, BrdUrd uptake was used to determine the relative percentage of cells in S phase. As expected from the findings from freshly isolated thymocytes using PI (Fig. 6), we find that in vivo BrdUrd uptake is significantly increased in Lck<sup>pr</sup>-Bax transgenic mice (Fig. 7, A and B). Furthermore, both the small thymus and the increased BrdUrd uptake occurred independently of p53; inasmuch as they were both observed in p53-deficient mice (Fig. 7, C and D). We conclude that Bax overexpression results in increased DNA synthesis or S-phase, and before malignancy, the thymic lymphopenia and proliferative changes are p53 independent.

**DISCUSSION**

The demonstration that human BAX can be a transcriptional target of p53 led to a model where BAX is a downstream effector of p53-dependent apoptosis (6). As p53 is the most frequently mutated gene in human tumors, Bax becomes a plausible candidate for a role in oncogenesis. A number of studies in mice support an important role of BAX in suppressing tumor development. BAX-deficiency potentiates tumor development in both SV40 TAg (8, 9) and E1A (10) transfection. However, these observations on the rule of Bax are in the context of a potent viral oncogene (adenovirus E1A or SV40 T antigen).

In this study Bax-deficiency alone (Fig. 2A) or in conjunction with p53-deficiency did not accelerate tumor development. The Bax-deficient mice showed decreased overall survival, but this could not be attributed to tumor development. Two of forty-one Bax-deficient animals died at <6 months of age and were found to have glomerulonephritis. However, given the low prevalence of glomerulonephritis, it does not appear to account entirely for the decreased survival of Bax-deficient mice.

In contrast with Bax deficiency, BCL-2 overexpression accelerates oncogenesis in p53-deficient mice. This observation extends previous work in mice that shows BCL-2 alone or in combination with MYC can promote tumor formation (5, 35). However, in a myc-induced model of hepatocellular carcinoma, BCL-2 inhibited tumor formation (36). BCL-2 inhibition of tumor formation was correlated with decreased proliferation in this model. Because Lck<sup>pr</sup>-Bax<sup>−/−</sup> also inhibits proliferation of T cells (25), the ability of Bcl-2 to cooperate with p53-deficiency and accelerate lymphoma formation was not obvious. Our findings show that when combined with p53 deficiency, the anti-apoptotic effects of BCL-2 dominate the anti-proliferative activity in regulating lymphoma formation. These results are consistent with the observation that BCL-2, but not p53-deficiency, inhibits apoptosis of T cells in response to diverse apoptotic signals (24).

Analysis of the Bax promoter suggests the p53 binding sites may not be functional in mice (29). Consistent with this finding, and in contrast to a previous result (37), we noted no differences in the levels of Bax in tissues derived from p53<sup>−/−</sup> and +/+ mice. However, regulation of murine Bax by p53 has been demonstrated in cells or tissues that also express either the TAg or E1A.

To further study the role of Bax in p53 mediated oncogenesis, mice overexpressing Bax in developing T cells were generated. Similar to transgenic mice that overexpress Bax using the CD2 promoter (33), our Lck<sup>pr</sup>-Bax transgenic mice display T-cell apoptosis (Fig. 3). In contrast with the CD2 promoter model, mice overexpressing Bax driven by the Lck<sup>pr</sup>-proximal promoter show marked thymic hypoplasia. Contrary to transient transfection of Bax into p53-deficient fibroblasts (10), overexpression of Bax in thymocytes promotes death to the same extent independent of the level of p53 (Fig. 5A). Moreover, CD2-Bax transgenic mice (33) and Lck<sup>pr</sup>-Bax transgenic mice are unable to confer sensitivity to γ-irradiation (Fig. 5B). Coupled with the observation that Bax-deficient thymocytes are not protected from γ-irradiation, these results demonstrate that Bax is neither necessary nor sufficient for apoptosis of thymocytes after γ-irradiation.

The most surprising finding was the accelerated tumor progression in Lck<sup>pr</sup>-Bax<sup>/p53</sup>-deficient mice. These differences were seen whether tumor-free or lymphoma-free survival was examined. These findings are potentially even more remarkable inasmuch as the “target” population of thymocytes is substantially reduced from the overexpression of BAX (Fig. 3A). Our paradoxical findings are reminiscent of...
E2F-1, in which the deficiency of this critical positive regulator of cell division results in tumor induction (38).

A number of possibilities may explain our paradoxical findings regarding Bax and tumor progression. We have no evidence that BAX would have a paradoxical anti-apoptosis effect in thymocytes. However, it is important to acknowledge several reports in neuronal systems, including transient Bax expression (31), Nigericin-induced apoptosis in follicular lymphoma (39), and the infection of Bax-deficient mice with a neurotropic virus (32) suggest that Bax may exert an anti-apoptotic effect. However, these examples of a potential anti-apoptotic activity for BAX were all in neuronal cells, and the applicability of these findings to lymphoid cells is not clear. In fact, before malignant transformation, Bax promotes apoptosis of thymocytes in the presence or absence of p53 (Fig. 3B).

A more likely explanation is that BAX-increased proliferation of thymocytes promotes transformation. In support, a number of studies have implicated BCL-2 in the regulation of cell cycle. Studies of T cells indicate that BCL-2 levels help dictate cell cycle entry from the quiescent state (25, 40, 41). These findings have been extended to other cell types, including NIH 3T3 fibroblasts (40) and IL-3-dependent hematopoietic cell lines (42). Other studies of BCL-2 suggest that inhibition of apoptosis is separable from cell cycle effects because two distinct mutations in BCL-2 result in normal anti-apoptotic function but the loss of anti-proliferation activity (42, 43). Downstream regulators of cellular proliferation appear to be altered in cells overexpressing BCL-2. BCL-2 decreased nuclear factor of activated T cells activation in mature T cells (25). Other studies demonstrate that BCL-2 affects p27Kip1 and p130 levels and the cyclin-dependent kinase 2 activity (44, 45). However, the biochemical activity of BCL-2 that accounts for these downstream changes remains to be elucidated.

Unfortunately, less is known concerning a role for BAX in cell cycle regulation. This reflects the difficulty of assessing cell cycle regulations in cells committed to die. Cells from the CD2-Bax transgenic mice do display accelerated entry into the cell cycle after ConA stimulation (34). In addition, thymocytes overexpressing Bax more rapidly degrade p27Kip1 and increase CDK2 kinase activity (44). These findings are in agreement with our observation that BAX-induced changes in proliferation and apoptosis occur simultaneously (Fig. 6B). This interplay of BAX effects on cell cycle and apoptosis is likely central to its unanticipated acceleration of tumorigenesis in the absence of p53.

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