Loss of Bax Alters Tumor Spectrum and Tumor Numbers in ARF-deficient Mice

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ABSTRACT

p19ARF is a key regulator of the p53-mediated apoptotic and tumor suppressor pathway. The proapoptotic Bax gene is a transcription target of p53, yet genetic studies in some animal models have suggested that Bax and p53 loss may cooperate in tumorigenesis. ARF-deficient mice are tumor prone, and to determine whether Bax loss could cooperate in the development of these tumors, we generated mice null for both ARF and Bax. The tumor latency of Bax
to ARF-/-, Bax
to ARF-/-, and Bax
to ARF-/- mice was similar with a mean survival of 48.9, 48.1, and 47.6 weeks, respectively. In Bax
to ARF-/- mice, the predominant tumor type was B- and T-cell lymphoma followed by sarcomas and a lack of carcinomas. However, the frequency of lymphoma development dramatically decreased, whereas that of sarcomas and carcinomas increased, in a gene dosage-dependent manner in Bax
to ARF-/- and Bax
to ARF-/- mice. Furthermore, uncommon tumors of ARF-/- mice (osteosarcoma and hemangiosarcoma) were observed in Bax/ARF-double null mice, and tumor types not described previously in ARF-null mice (mixed germ cell tumor, Triton tumor, and histiocytic sarcoma) also developed in Bax
to ARF-/- animals. Importantly, multiple primary malignant tumors of different lineage arose in 25% of the Bax
to ARF-/- mice, whereas only one tumor type per animal was observed in Bax
to ARF-/- littermates. Finally, the wild-type Bax allele was retained in tumors arising in Bax
to ARF-/- mice. Thus, Bax appears to function as a tumor modifier rather than as a classic tumor suppressor, and the combined loss of Bax and the ARF allows for the emergence of multiple malignant tumor types, an alteration of the tumor spectrum, and tumors not observed previously in ARF-null mice.

INTRODUCTION

Cells that have sustained excessive genetic damage or are exposed to inappropriate proliferative signals undergo apoptosis that is dependent on p53 and/or ARF (1–6). One function of ARF is to regulate p53 activity by binding to and sequestering the p53 regulator Mdm2, and p53 suppressor activities of ARF and p53 were established in mice lacking ARF or p53, which spontaneously develop tumors with a 100% penetrance (13, 14). ARF-deficient mice on a C57Bl/6 × 129SVj mixed background preferentially develop undifferentiated sarcomas (43%), followed by T-cell lymphomas (29%), carcinomas (17%), and tumors of the nervous system (11%), with an average survival of 38 weeks (14, 15). By contrast, p53-null mice preferentially develop T-cell lymphomas (70%), followed by fibrosarcomas (30%), and have a mean survival of ~19 weeks (13, 16). Therefore, inactivation of ARF or p53 is a critical step in tumor development, but their loss seems to have context-specific effects on tumorigenesis.

The Bcl-2 family of proteins includes both antiapoptotic and proapoptotic members that are essential for regulating cell survival (reviewed in Ref. 17), and these have also been linked to tumor development (reviewed in Ref. 18). The antiapoptotic proteins Bcl-2 and Bcl-xL are overexpressed in a wide variety of human and murine malignancies (18–20), whereas inactivating mutations in the proapoptotic bax and bak genes have been reported in colon cancers and other cancer types (21–26). Despite these findings, the overexpression of antiapoptotic Bcl-2 family members or inactivation of proapoptotic members alone or rarely results in cancer (27–30), e.g., unlike p53- and ARF-deficient mice, Bax-/-, Bax-, and Bax/Bak-double null mice do not develop spontaneous tumors (29, 30). Additionally, mice engineered to overexpress Bcl-2 have a very low incidence of malignancy (27, 28).

When combined with oncogenes or loss of tumor suppressors, alterations in Bcl-2 family members cooperate to enhance transformation and accelerate tumorigenesis, e.g., E6/7-cbl-2/E6/7-myc double transgenic mice develop lymphoma much more rapidly than mice expressing either transgene alone (31). Furthermore, Bax-null MEFs are more susceptible to transformation than wild-type MEFs (32). Finally, loss of Bax accelerates brain and breast tumor development in SV40 large T antigen transgenic mice (33, 34), suggesting that Bax loss can collaborate with inactivation of p53 and/or Rb in tumorigenesis. Cooperation of Bax loss with the Rb pathway appears more likely, as Bax is a transcription target of p53 (35), and Bax loss does not alter the tumor spectrum or tumor latency in p53-null mice (36). In contrast, here we establish that Bax loss cooperates with ARF loss in tumorigenesis. Mice lacking both ARF and Bax have a marked alteration in their spectrum of tumors compared with those that arise in ARF-null mice, without altering the mean tumor latency. Furthermore, Bax/ARF-double null mice develop uncommon tumors and multiple primary malignancies, which are not observed in ARF-null mice. These results support the concept that there are cooperative effects of tumor suppressor loss and alterations in the Bcl-2 family of apoptotic regulators on tumor development.

MATERIALS AND METHODS

Mice. Both ARF- and Bax-null mice were generated using RW4 embryonic stem cells (14, 29). ARF-null mice (C57Bl/6 × 129SVj) were kindly provided by Dr. Charles J. Sherr. Bax heterozygous mice (C57Bl/6 × 129SvJ) were crossed to ARF-null mice, and the F1s were then interbred to generate F2 Bax
to ARF-/-, Bax
to ARF-/-, and Bax
to ARF-/- mice. All F2 littermates

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4 The abbreviations used are: MEF, mouse embryo fibroblast; Rb, retinoblastoma MSA, muscle-specific actin; SMA, smooth muscle actin.

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were observed daily and immediately sacrificed when signs of illness were detected. Mice were necropsied, and tissues were collected and formalin fixed (see below) for histological analysis. Kaplan-Meier analysis was performed on the time of tumor latency of the three genotypes of ARF-null mice.

**Histological Analyses.** Tissues from sacrificed or recently deceased animals were collected and fixed in 10% buffered formalin and embedded in paraffin. Classification of tumors was determined on fixed tissue sections using light microscopy and standard immunohistochemistry techniques. Lymphomas were classified as T-cell lymphoma, B-cell lymphoma, polymorphic lymphoma, or lymphoma of indeterminate lineage. In T-cell lymphoma, ≥75% of the tumor cells expressed the T-lymphocyte CD3 antigen, and in B-cell lymphoma, ≥75% of the tumor cells expressed the B-lymphocyte CD19 (B220) antigen. In polymorphic lymphoma, there was a mixture of malignant lymphocytes expressing the CD3 or B220 antigen, as well as a mixture of myeloid cells consisting of histiocytes expressing the Mac2 antigen, neutrophils, eosinophils, and an occasional multinucleated giant cell. In lymphoma of indeterminate lineage, the majority of the tumor cells stained negative for CD3, CD20, and Mac2.

Heat-induced epitope retrieval was performed in a Black and Decker Steamer with citrate buffer (pH 6.0) at 95°C for 15–30 min for CD3, CD4, CD45R/B220, MAC2, Factor VIII, S-100, actin, desmin, SMA, and myogenin. A 10-min proteinase K digestion treatment was used for the cytokeratin, CD31, and lysozyme assays. Endogenous peroxidase activity was inactivated with H2O2.

The following antibodies were purchased from DAKO (Carpinteria, CA): mouse antihuman muscle actin (MSA, clone HHF35), mouse antihuman α SMA (clone 1A4), mouse antihuman desmin (clone D3), mouse antimitogen (clone F5D), rabbit antihuman CD3, rabbit antivimentin cytokeratin wide spectrum, rabbit antihuman Factor VIII, rabbit antihuman lysozyme, rabbit antihuman myeloperoxidase, rabbit antivimentin S-100, mouse IgG1k, and rabbit immunoglobulins. Mouse antihuman cytokeratin (clones AE1 and AE3) was obtained from Chemicon International, Inc. (Temecula, CA). Rat antimonocle D31 (clone MEC 13.3), rat antimouse CD34 (clone RAM34), rat antimouse CD45R/B220 (clone RA3–62B), mouse IgG2a κ (clone 1B.18.4), rat IgG2a κ (clone R35–95), and rat IgG2b κ (clone A95–1) were obtained from PharMingen (San Diego, CA). Rat antimouse MAC2 (clone M3/38) was obtained from Accurate Antibodies (Westbury, NY). Biotinylated secondary antibodies goat antirabbit and rabbit antirat were obtained from Vector Laboratories, Inc. (Burlingame, CA). Negative controls were prepared by omitting primary antibody and substituting isotype-matched IgGs at equivalent concentrations. After the streptavidin-biotin-peroxidase complex method for antigen visualization, the tissue sections were counterstained with hematoxylin (DAKO), dehydrated, and coverslipped.

**Bax PCR Analysis.** A 3–5-mm piece of formalin-fixed, paraffin-embedded tumor from each of the Bax+/– ARF–/– mice listed on Table 1 was cut from each block. Genomic DNA was isolated from the formalin-fixed, paraffin-embedded tissues as described elsewhere (Method A; Ref. 37). Each digested sample (0.1–10 μL) was used for Bax PCR analysis. Primers and conditions reported previously (29) were used to amplify a 304-bp Bax wild type and a 507-bp Bax knockout band.

**RESULTS**

**Tumor Latency in Bax+/– ARF–/– Mice.** SV40 large T antigen inactivates p53 (reviewed in Ref. 38), and Bax loss in SV40 large T antigen transgenic mice accelerates brain and breast tumor development (33, 34). However, SV40 large T antigen also inactivates Rb, and loss of Bax does not alter tumor formation in p53-null mice (36). ARF and p53 function shared a tumor suppressor pathway (reviewed in Ref. 39), yet ARF has p53-independent functions (40), and p53 can be activated independent of ARF (14). To determine whether Bax loss cooperates with ARF loss in tumorigenesis, we generated Bax/ARF−/− double null mice and followed them for tumor development. It was reported previously that ARF-null mice on a mixed C57Bl/6 × 129SvJ background have an average survival of 38 weeks (14, 15), whereas F2 ARF-null mice that we crossed onto the Bax background (a different mixed C57Bl/6 × 129SvJ background) have an average survival of 49 weeks (Fig. 1). Importantly, tumors arose at comparable intervals in Bax+/– ARF−/–, Bax+/– ARF+/–, and Bax−/– ARF+/– mice, which had a mean survival of 48.9, 48.1, and 47.6 weeks, respectively (Fig. 1). Tumors were identified in all mice. Therefore, Bax loss does not influence the overall rate of tumor development or survival in ARF-null mice.

**Lymphoma Development in Bax+/– ARF–/– Mice.** Tumor latency is only one measure of whether a gene affects tumorigenesis, and tumor spectrum can also reveal cooperativity between genes, e.g., a third of ARF/p53-double null mice develop multiple primary tumors (40), whereas only one tumor type emerges in mice lacking p53 or ARF alone (13–16). This suggests cooperation between ARF and p53 inactivation, although both contribute to the tumor suppressor pathway activated by oncogenes (5, 6). It was reported previously that ARF-null mice on a mixed C57Bl/6 × 129SvJ background develop sarcomas (43%), followed by T-cell lymphomas (29%), carcinomas (17%), and tumors of the nervous system (11%; Refs. 14 and 15). Unexpectedly, lymphoma was the most common spontaneously arising tumor in all ARF-null mice generated from the Bax crosses. The majority of the lymphomas were of either B- or T-cell lineage (Table 1; Fig. 2A). T-cell lymphomas occurred in 38% (8 of 21) of Bax+/– ARF−/– mice (Table 1), which is similar to the percentage reported previously (14, 15). In contrast, B-cell lymphomas were rare.

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**Table 1 Tumor spectrum in Bax+/– ARF−/–, Bax+/– ARF+/–, and Bax−/– ARF+/– mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Tumor type(s)</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax+/– ARF−/–</td>
<td>B-cell lymphoma</td>
<td>7</td>
</tr>
<tr>
<td>(21 mice)</td>
<td>T-cell lymphoma</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>B-cell and T-cell lymphoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Polymorphic lymphoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Lymphoma of indeterminate lineage</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Neural sarcoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal stromal tumor of neural type</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle sarcoma</td>
<td>1</td>
</tr>
<tr>
<td>Bax+/– ARF+/–</td>
<td>B-cell lymphoma</td>
<td>4</td>
</tr>
<tr>
<td>(21 mice)</td>
<td>T-cell lymphoma</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Polymorphic lymphoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Lymphoma of indeterminate lineage</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Neural sarcoma</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Histiocytic sarcoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle sarcoma</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Squamous cell carcinoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Neural sarcoma + pulmonary carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Bax−/– ARF+/–</td>
<td>B-cell lymphoma</td>
<td>4</td>
</tr>
<tr>
<td>(25 mice)</td>
<td>T-cell lymphoma</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Polymorphic lymphoma</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Lymphoma of indeterminate lineage</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Neural sarcoma</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Smooth muscle sarcoma</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Osteosarcoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Triton tumor</td>
<td>1</td>
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<tr>
<td></td>
<td>Mixed germ cell tumor</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B-cell lymphoma + sarcoma of indeterminate lineage</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B-cell lymphoma + smooth muscle sarcoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>B-cell lymphoma + histiocytic sarcoma</td>
<td>1</td>
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<td></td>
<td>T-cell lymphoma + histiocytic sarcoma</td>
<td>1</td>
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<tr>
<td></td>
<td>Polymorphic lymphoma + hemangiosarcoma</td>
<td>1</td>
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<tr>
<td></td>
<td>Histiocytic sarcoma + pulmonary carcinoma</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Histiocytic sarcoma + intestinal carcinoma</td>
<td>1</td>
</tr>
</tbody>
</table>

*Number of mice of each genotype analyzed are indicated in parentheses below each genotype.
*One Bax+/– ARF−/– (squamous cell papilloma) and one Bax−/– ARF−/– (hemangioma) that only had benign tumors were excluded from this list.
*One mouse had a benign hemangioma in addition to polymorphic lymphoma.
*One mouse had a benign spindle cell tumor in addition to B-cell lymphoma.
*Bold font indicates animals that developed two primary malignant tumors of different lineage.
tumors of both lymphoid and myeloid hematopoietic cell origin, or a lymphoma with a mixed cellular host reaction, multiple primary antigen were classified as polymorphic lymphomas (Table 1). A low tions that expressed CD3, B220, and the myeloid cell-specific Mac2 antigen CD3 (data not shown). Tumors with cells of variable propor-
tions of each genotype that were analyzed are indicated in Fig. 2. Loss of Bax Alters the Tumor Spectrum of ARF-null mice. After lymphomas, the second most common tumor type observed in all ARF-null mice (Bax+/−, Bax+/, and Bax−−) was sarcoma (Table 1). The percentage of Bax−− ARF−− mice that developed sarcomas with non-neural differentiation, 44% (11 of 25; Fig. 2A), was virtually identical to the percentage reported previously for ARF-null mice (43%; Refs. 14 and 15). Sarcomas arising in animals from the ARF X Bax cross included histiocytic sarcoma (Fig. 3B), smooth muscle sarcoma (Fig. 3C), osteosarcoma, and hemangiosarcoma (Fig. 3D). The smooth muscle sarcomas (Table 1) expressed SMA and SM (Fig. 3C, inset) but were negative for desmin, myogenin, and S-100 (data not shown).

Interestingly, a greater percentage of Bax−− ARF−− mice developed non-neural sarcomas than Bax+/− ARF−− mice (Table 1; Fig. 2A). Bax gene dosage affected the frequency of these sarcomas, 44% (11 of 25) in Bax/ARF-double null mice, 14% (3 of 21) in Bax+/− ARF−− mice, and 5% (1 of 21) in Bax+/− ARF−− mice (Fig. 2A). Sarcomas usually arise in older mice, so we assessed whether Bax−− ARF−− mice that developed these sarcomas survived longer. However, this was not the case, as 42% of Bax−− ARF−− and 50% of Bax+/− ARF−− survived for >12 months. Thus, Bax−− ARF−− mice are more predisposed to developing non-neural sarcomas, suggesting that loss of Bax increases the susceptibility of ARF-null mice to these tumor types.

Neural sarcomas developed at a similar frequency in all genotypes (Bax+/+, Bax+/−, and Bax−−) of ARF-null mice [i.e., 10 (2 of 21), 14 (3 of 21), and 12% (3 of 25) of the mice, respectively; Table 1; Fig.
Carcinomas were more rare, as only 10 and 8% of the tumors from Bax<sup>+/−</sup>ARF<sup>−/−</sup> and Bax<sup>−/−</sup>ARF<sup>−/−</sup> mice, respectively, had this tumor type (Table 1; Fig. 2A). Pulmonary carcinoma developed in both Bax<sup>+/−</sup>ARF<sup>−/−</sup> and Bax<sup>−/−</sup>ARF<sup>−/−</sup> mice (Fig. 3F), whereas an intestinal carcinoma in situ was observed in a Bax<sup>−/−</sup>ARF<sup>−/−</sup> mouse (Fig. 3E), and a squamous cell carcinoma was identified in a Bax<sup>+/−</sup>ARF<sup>−/−</sup> mouse (data not shown). Carcinomas did not develop in any Bax<sup>+/−</sup>ARF<sup>−/−</sup> mice. In the original strain of ARF-null mice, carcinomas were slightly more frequent (17% of mice analyzed; Refs. 14 and 15), and this difference may again be because of genetic background effects. Nevertheless, the loss of Bax increases the frequency of carcinoma development in the ARF-null mice in this study. Overall, the tumor spectrum in ARF-null mice had marked shifts in response to Bax loss with many fewer lymphomas and more sarcomas and carcinomas, without significantly altering the frequency of neural tumors.

**Multiple Primary and Unique Tumors Arise in Bax<sup>−/−</sup>ARF<sup>−/−</sup> Mice.** An unpredicted result of this cross was that more than one primary malignant tumor type of different lineages developed in several Bax<sup>−/−</sup>ARF<sup>−/−</sup> mice and in one Bax<sup>+/−</sup>ARF<sup>−/−</sup> mouse. This is highly unusual, as ARF-null mice develop only one primary tumor type, unless treated with chemical carcinogens (14, 15) or when crossed into a p53-null background (40), e.g., multiple primary tumors occurred in 46% (6 of 13) of ARF-null mice subjected to dimethylbenzanthrene (14) and in 28% (5 of 18) of ARF/p53-double null and 47% (9 of 19) of ARF/Mdm2/p53-triple null mice (40). We determined that 28% (7 of 25) of Bax<sup>−/−</sup>ARF<sup>−/−</sup> mice developed both a sarcoma (smooth muscle sarcoma, sarcoma of indeterminate lineage, or histiocytic sarcoma) and a lymphoma or a carcinoma (pulmonary or intestinal; Table 1; Fig. 2B). The histiocytic sarcomas in two mice that also developed lymphomas involved the liver, whereas the lymphomas involved the lymphoid organs (Fig. 3A). A smooth muscle sarcoma and a B-cell lymphoma, and a sarcoma of indeterminate
lineage and a B-cell lymphoma, occurred in two Bax<sup>−/−</sup> ARF<sup>−/−</sup> mice (Table 1). Both a histiocytic sarcoma and a pulmonary carcinoma (Fig. 3F), and a histiocytic sarcoma and an intestinal carcinoma in situ involving the small intestinal villi (Fig. 3E), emerged in two different Bax<sup>−/−</sup> ARF<sup>−/−</sup> mice (Table 1). Only one Bax<sup>−/+</sup> ARF<sup>−/−</sup> mouse developed multiple primary malignant tumors (Table 1). Therefore, Bax loss also predisposes ARF-null mice to transforming events that result in multiple primary malignant tumors developing in a single mouse.

Tumors that appear uncommon in ARF-null only animals were also observed in Bax/ARF-double null mice (Table 1). An osteogenic and a hemangiosarcoma (Fig. 3D) mixed germ cell tumor originated in the testes and was comprised of a teratoma and an embryonal carcinoma (Fig. 4, B, D, and F). However, this tumor also had mononuclear giant cells with cytological morphology of trophoblasts, and was consequently classified as a mixed germ cell tumor. The Triton tumor was a spindle cell tumor that transversed through skeletal muscle, bone, and nerves (Fig. 4, A, C, and E). Finally, Bax loss also results in the emergence of histiocytic sarcomas in ARF-null mice (Fig. 3B). Histiocytic sarcomas developed in 16% (4 of 25) of Bax<sup>−/−</sup> ARF<sup>−/−</sup> and 5% (1 of 21) of Bax<sup>−/+</sup> ARF<sup>−/−</sup> mice, and none were observed in Bax<sup>−/+</sup> ARF<sup>−/−</sup> mice (Table 1). Therefore, loss of both Bax and ARF allows for the emergence of rare malignancies and tumors not described for ARF-null mice.

**Bax Is a Tumor Modifier.** Although Bax-null mice do not develop spontaneous tumors (29), these results suggested that Bax might act as a tumor suppressor, particularly in cooperation with ARF loss. One prediction was that if Bax is a “classic” tumor suppressor, as suggested by others (33, 34), then at least a fraction of the Bax<sup>−/−</sup> ARF<sup>−/−</sup> tumors should lose the remaining wild-type Bax allele. However, analysis of genomic DNA isolated from tumor-arising Bax<sup>−/−</sup> ARF<sup>−/−</sup> mice revealed that none of these tumors lost the wild-type allele of Bax (Fig. 5). Therefore, Bax does not appear to act as a classic tumor suppressor but rather appears to function as a tumor modifier in most malignancies, including those that arise in Bax<sup>−/−</sup> ARF<sup>−/−</sup> mice where haplo-insufficiency clearly affects tumorigenesis.

**DISCUSSION**

Here we have established that there are cooperative effects of the loss of the apoptotic mediator Bax and the tumor suppressor ARF in tumor development. Despite a similar tumor latency and survival of Bax<sup>−/+</sup> ARF<sup>−/−</sup> and Bax<sup>−/−</sup> ARF<sup>−/−</sup> mice, there were marked alter-
mixed germ cell tumor, the Triton tumor, and the histiocytic sarcomas. Although the different genotypes in the frequency of neural sarcomas. Nevertheless, in our study, 86% of multiple primary malignant tumors (a lymphoma and a sarcoma or carcinomas) were observed (40). These tumors had not been described in ARF-null mice (14, 15), just as the mixed germ cell tumor, the Triton tumor, and the histiocytic sarcomas that developed in Bax−/−ARF−/− mice appear unique. In addition, multiple primary malignant tumors (a lymphoma and a sarcoma or carcinoma) arose in a third of mice lacking p53 and ARF and in half of the mice deficient in p53, ARF, and Mdm2 (40). A portion of the Bax−/−ARF−/− mice (28%) developed two different primary malignant tumor types; a sarcoma plus a lymphoma was the most common, but a sarcoma and a carcinoma occurred in 8% of Bax/ARF-double null mice. Thus, the loss of multiple genes that regulate cell growth and/or survival appears to lead to a greater frequency and diversity of tumors.

There was a striking decrease in the percentage of lymphomas, and an increased frequency of non-neural sarcomas and carcinomas, on loss of both Bax and ARF, whereas no difference was seen between the different genotypes in the frequency of neural sarcomas. Although the strain background of the Bax X ARF cross did appear to alter the tumor spectrum, the effects of Bax loss were clearly evident. It was reported previously that sarcomas, lymphomas, and carcinomas emerge in 43, 29, and 17%, respectively, of ARF-null mice (14, 15). However, in our study, 86% of Bax+/+ARF+/+ mice developed lymphomas, followed by sarcomas (5%), and no carcinomas were observed. A similar shift in the tumor spectrum was observed in ARF−/−p53−/− mice, where lymphomas emerged in 72% (13 of 18) and sarcomas developed in 22% (4 of 18) of the ARF/p53-double-null mice (40). However, it was not reported whether ARF−/−p53−/− littermate controls also preferentially developed lymphomas over sarcomas; therefore, it is unclear whether this was because of a background effect or to just loss of p53, which normally results in lymphomas in 70% of p53−/− mice (13, 16). The lymphomas that did emerge in ARF/p53-double null and ARF/p53/Mdm2-triple null mice were of T-cell lineage with one exception (40), which is consistent with the lymphoma type that p53-null mice acquire, whereas B- and T-cell lymphomas developed with equal frequency in Bax+/+ARF−/− mice. Interestingly, loss of Mdm2 in addition to a deficiency in ARF and p53 reduced the percentage of lymphomas that emerged from 72 (13 of 18) to 47% (9 of 19; Ref. 40). A decrease in lymphomas was also evident when Bax was lost, 86% (18 of 21) in Bax+/+ARF−/− to 60% (15 of 25) in Bax−/−ARF−/− mice. In addition, 22% (4 of 18) of ARF−/−p53−/− and 58% (11 of 19) of ARF−/−p53−/−Mdm2−/− mice developed sarcomas (40), suggesting a positive effect on sarcoma development by Mdm2 loss. We observed a similar effect on the frequency of sarcomas with Bax loss. Non-neural sarcomas emerged in 5% (1 of 21) of Bax+/+ARF−/− mice and 44% (11 of 25) in Bax−/−ARF−/− mice. Therefore, loss of regulators of cell survival, such as Mdm2 in an ARF/p53-null context (40) or Bax in an ARF-null context, appears to have similar effects on tumor spectrum and predisposes mice to the emergence of non-neural sarcomas over lymphomas.

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ARF and p53 both function as classic tumor suppressors and can function in a shared pathway. However, p53 can function independent of ARF and vice versa (14, 40, 45). Unlike Bax−/−ARF−/− mice, Bax−/−p53−/− mice are equivalent to p53-null mice in their tumor latency and tumor spectrum (36). This is in apparent contrast to a report indicating that inactivation of p53 by SV40 large T antigen and a deficiency in Bax accelerates breast tumor development (34). However, SV40 large T antigen also compromises the function of other tumor suppressors, in particular, the Rb pocket family of proteins (reviewed in Ref. 38), and transgenic mice expressing a truncated SV40 large T antigen that only inactivates Rb also show cooperativity with Bax loss in brain tumor development (33). Therefore, the lack of cooperation of Bax and p53 loss on tumorigenesis in Bax−/−p53−/− mice suggests that Bax and p53 reside in a common pathway. This is substantiated by reports indicating that at least some cell contexts, Bax is a direct transcription target of p53 (35) and is up-regulated by p53 expression (46) or in response to DNA damage (47). Furthermore, lymphomas that arise in Bax−/−Eμ-‐myc transgenic mice fail to sustain p53 mutations or deletions, yet the frequency of ARF deletion remains the same as in lymphomas derived from wild-type Eμ-‐myc transgensics (48). Thus, Bax and p53 appear to lie in a common pathway, whereas Bax and ARF seem to function in separate pathways.

Bax loss influences the ARF pathway in at least two respects, altering the tumor spectrum, including the types of tumors that develop, and allowing for the emergence of multiple primary tumors. In this scenario and others, the role of Bax is more consistent as that of a tumor modifier rather than as a classic tumor suppressor, as tumors that arise in Bax+/+ARF−/− mice (Fig. 5) or Bax+/+Eμ-‐myc transgensics (48) always retain the wild-type Bax allele. However, there are clear synergistic effects on tumorigenesis that occur when both Bax and ARF are absent, suggesting that Bax and ARF lie in parallel complementary pathways.

The findings that loss of Bax and ARF cooperates in tumorigenesis in mice suggest that a similar cooperation may also be involved in human cancers. Although direct comparisons of ARF and Bax loss have not been performed in human tumors, there are several tumor types where both have been implicated. BAX is mutated in many tumor types, especially those having the microsatellite mutator phenotype, including pancreatic adenocarcinomas, gastric and colon cancers, and in T-cell acute lymphoblastic leukemia (21–26). Strikingly INK4A/ARF is inactivated by methylation or deletion in these same tumor types (49–53). A second issue that certainly bears study in human cancer is whether malignancies comparable with Triton tumors, mixed germ cell tumors, and histiocytic sarcoma, which only arise in Bax/ARF double-null mice, also suffer mutations of BAX and loss of ARF. Finally, because the wild-type Bax allele is retained in tumors arising in Bax+/+ARF−/− mice and Bax+/−Eμ-‐myc transgensics (48), it will also be interesting to evaluate if there is loss of function of both BAX alleles in human tumors.

If Bax and ARF are in separate pathways, how does Bax loss cooperate with ARF loss? Inactivation of ARF results in immortalization of many cell types (e.g., MEFs, pre-B cells, and myeloid precursors; Refs. 5, 14, and 54), whereas Bax is an apoptotic mediator activated in response to a wide variety of apoptotic signals (reviewed in Ref. 17). One prediction would be that Bax loss confers resistance to certain apoptotic stimuli and, therefore, allows cells that would otherwise die to survive, e.g., MEFs and pre-B cells lacking Bax are...
more resistant to the apoptotic effects of the oncprotein Myc (48, 55), and there is less apoptosis seen in neural precursors lacking Bax that are exposed to γ-irradiation (56). Therefore, an immortalizing event, such as ARF inactivation, combined with an increased resistance to apoptosis from Bax loss, is a recipe for cancer development and progression. In support of this concept, cells that lack p53 function and overexpress the antiapoptotic Bcl-XL protein are genetically unstable and have a higher rate of polyploidization (57). Furthermore, a mutation of p53 in human follicular lymphomas that already overexpress Bcl-2 because of translocations is usually indicative of tumor progression and a poorer prognosis (58, 59). Many other cancer types, both of human and murine origin, also overexpress Bcl-2 and/or Bcl-X and disable ARF or p53 function (11, 12, 18).

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REFERENCES


Loss of Bax Alters Tumor Spectrum and Tumor Numbers in ARF-deficient Mice

Christine M. Eischen, Jerold E. Rehg, Stanley J. Korsmeyer, et al.


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