Haploid loss of *bax* leads to accelerated mammary tumor development in C3(1)/SV40-TAg transgenic mice: reduction in protective apoptotic response at the preneoplastic stage

Masa-Aki Shibata, Min-Ling Liu, Michael C.Knudson¹, Eiko Shibata, Katsuhide Yoshidome, Tabassum Bandey², Stanley J.Korsmeyer¹ and Jeffrey E.Green³

Laboratory of Cell Regulation and Carcinogenesis, Division of Basic Sciences, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, ¹Dana-Farber Cancer Institute, Harvard University, Boston, MA and ²Science Applications International Corporation, Frederick, MD, USA

³Corresponding author
e-mail: JEGreen@nih.gov

M-A.Shibata and M.-L.Liu contributed equally to this work

The dramatic increase in apoptosis observed during the development of preneoplastic mammary lesions is associated with a significant elevation in Bax expression in C3(1)/SV40 large T antigen (TAg) transgenic mice. The significance of Bax expression during tumor progression *in vivo* was studied by generating double-transgenic mice carrying the C3(1)/TAg transgene and mutant alleles for *bax*. C3(1)/TAg transgenic mice carrying mutant *bax* alleles exhibited accelerated rates of tumor growth, increased tumor numbers, larger tumor mass and decreased survival rates compared with mice carrying wild-type *bax*. Accelerated tumorigenesis associated with the *bax*^{+/-} genotype did not require the loss of function of the second *bax* allele. Thus, haploid insufficiency of *bax* is enough to accelerate tumor progression, suggesting that the protective effect of Bax is dose-dependent. While levels of apoptosis in the preneoplastic lesions, but not carcinomas, were reduced in *bax*^{+/-} or *bax*^{-/-} mice compared with *bax*^{+/+} mice, rates of cellular proliferation in mammary lesions were similar among all *bax* genotypes. These data demonstrate that *bax* is a critical suppressor of mammary tumor progression at the stage of preneoplastic mammary lesion development through the upregulation of apoptosis, but that this protective effect is lost during the transition from preneoplasia to invasive carcinoma.

Keywords: apoptosis/*bax*/cell death/mammary gland/transgenic mice/tumor progression

Introduction

Human breast cancer has become the second leading cause of cancer deaths in women in the USA and its incidence continues to rise (Harris *et al.*, 1992). Since there are numerous limitations to the study of human breast cancer, we have taken advantage of the unique C3(1)/SV40 large T antigen (TAg) transgenic mouse model of mammary cancer (Maroulakou *et al.*, 1994; Shibata *et al.*, 1996), which shares many important similarities with human

breast cancer, to study genes involved in regulating the changes in apoptotic response observed during different stages of mammary tumor progression.

Apoptosis is a genetically determined process resulting in cell death in response to various stimuli which serves to eliminate excessive or damaged cells. Apoptosis is thought to be a critical defense mechanism for eliminating cells that have been damaged or have acquired genetic defects which may lead to the development of cancer. The dysregulation of the normal process of apoptotic cell death may result in pathological accumulations of cells and contribute to the development of hyperplastic and neoplastic lesions (White, 1996). We have reported previously that there is a large increase in levels of apoptosis in preneoplastic mammary lesions in the C3(1)/TAg mice which is dramatically reduced during the transition to invasive carcinoma (Shibata *et al.*, 1996). This loss of apoptotic response appears to be a critical determinant of tumor progression in this model.

Members of the *bcl-2* family of genes are key regulators of apoptosis. A number of genes that share sequence homology with *bcl-2* have been isolated. Bcl-2 family members and their associated proteins display either positive or negative regulatory effects on apoptosis. Bcl-2 and Bcl-X_L block apoptosis (Hockenbery *et al.*, 1990; Boise *et al.*, 1993), whereas other members of the Bcl-2 family such as Bax, Bcl-X_S and Bak, promote apoptotic cell death (Boise *et al.*, 1993; Oltvai *et al.*, 1993; Chittenden *et al.*, 1995; Kiefer *et al.*, 1995). Bax heterodimerizes with Bcl-2 or Bcl-X_L and results in inhibition of their anti-apoptotic activity (Yin *et al.*, 1994). Bax may, however, upregulate apoptosis independently of heterodimerizing with proteins such as Bcl-2 or Bcl-X_L (Gross *et al.*, 1998).

In addition to the Bcl-2 family, the *p53* tumor suppressor gene, the most frequently mutated gene in human tumors (Greenblatt *et al.*, 1994), has been recognized to be an important mediator in apoptotic signaling. Transfection of *p53* into transformed cells can induce either growth arrest (El-Deiry *et al.*, 1993; Harper *et al.*, 1993) or apoptosis (Yonishi-Rouach, 1991). One mechanism for *p53*-induced apoptosis has been through the induction of Bax by *p53*. *p53* has been shown to stimulate Bax expression through transcriptional activation using promoter-reporter assays *in vitro* (Miyashita and Reed, 1995). However, overexpression of Bax induces apoptosis where *p53* is mutant, suggesting that Bax alone is capable of inducing apoptosis and that Bax functions downstream of *p53* (Han *et al.*, 1996).

The role of Bax during oncogenesis has not been well studied and may vary according to tissue type. One-third of advanced human breast carcinomas demonstrate a marked reduction in Bax expression as assessed by immunohistochemistry (Krajewski *et al.*, 1995). A pre-

vious study has demonstrated that p53-dependent Bax expression suppresses tumor development in a transgenic mouse brain tumor model (Yin *et al.*, 1997). In this study, we determined that increased apoptosis during the early stage of mammary cancer development in the C3(1)/Tag transgenic mouse model is associated with an elevation in Bax expression. This induction of Bax is functionally significant in mammary cancer progression as we demonstrate that the loss of just one *bax* allele leads to enhanced mammary cancer development and tumor growth. Thus, *bax* does not function as a typical tumor suppressor gene, but appears only to require haploid insufficiency to alter tumor growth. Accelerated mammary cancer growth results from a significant reduction in the apoptotic response, which is limited to the preneoplastic stage of tumor development in mice carrying the C3(1)/Tag transgene and mutant *bax* alleles. Bax-induced apoptosis is independent of the functional status of p53 in this model.

Results

Apoptosis and cell proliferation during mammary tumor progression in C3(1)/Tag transgenic mice carrying wild-type *bax* alleles

The course of mammary tumor development in C3(1)/Tag transgenic mice is highly predictable (Shibata *et al.*, 1996) and correlates well with progressive stages of human breast cancer. All transgenic female mice develop atypical ductal hyperplasias (AH) by 8 weeks of age, nodular atypical ductal hyperplasias (NAH) by 12 weeks of age (Figure 1A) and invasive carcinomas (Figure 1B) by 16–20 weeks of age. The lesions, which we define as AH and NAH in the transgenic mice, are regarded as preneoplastic lesions (Shibata *et al.*, 1996). Apoptotic cells were identified in the various mammary lesions (excluding regions of tumor necrosis) using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) assay. The identification of apoptotic cells was further confirmed by ultrastructural analysis which demonstrated fragmented nuclei with pyknotic chromatin and nuclear fragments which occasionally contained a characteristic half-moon of condensed chromatin (Figure 1E and F).

Sequential changes in levels of apoptosis were quantitated during mammary tumor progression of the C3(1)/Tag transgenic mice carrying wild-type *bax* alleles (Figure 1C and D). Alterations in levels of apoptosis during sequential stages of tumor development are illustrated in Figure 1G. Levels of apoptosis in normal mammary epithelium were low at all ages. All mammary lesions, regardless of ages, showed elevation in apoptosis compared with normal epithelium. The levels of apoptosis were most dramatically elevated in AH and NAH at 12–16 weeks of age. However, apoptosis levels in NAH at 20 weeks of age were markedly reduced. This is the stage where the transition from NAH to invasive carcinoma occurs. This suggests that although the histological features of the NAH lesions at 12 and 20 weeks of age are similar, genetic changes may be occurring which contribute to the transition from NAH to invasive carcinoma. Levels of apoptosis were also significantly reduced in invasive carcinomas.

Sequential changes in proliferative cell nuclear antigen (PCNA) expression in different lesions of the mammary glands are illustrated in Figure 1H. A low percentage of PCNA immunoreactivity was observed in normal mammary epithelial cells at all ages. The percentage of PCNA-immunoreactive cells in all mammary lesions was significantly increased compared with that of normal epithelium. The percentage of PCNA-positive cells correlates with the degree of severity of the mammary lesions.

Expression of Bcl-2 family members in C3(1)/Tag mammary lesions carrying wild-type *bax* alleles

Northern blot, Western blot and immunohistochemical analyses were performed to determine expression levels of Bcl-2, Bax, Bak, Bcl-X_S and Bcl-X_L at different stages of mammary tumor progression. *bax* mRNA (1.0-kb α and 1.5-kb β forms) and protein (21 kDa) were markedly increased in preneoplastic lesions and carcinomas (Figure 2A and B), as was the short (~20 kDa) splice variant of Bcl-X, Bcl-X_S (Figure 2B). Immunohistochemical analyses confirmed that Bax was overexpressed in the AH, NAH and neoplastic lesions (Figure 2C–E). No *bcl-2* mRNA (data not shown) or protein (Shibata *et al.*, 1996) was detected in mammary lesions at any stage of tumor development, whereas the levels of Bcl-X_L (~29 kDa), the long splice variant of *bcl-X*, did not change during mammary tumor progression (Figure 2B). Immunostaining failed to reveal any difference in total Bcl-X_{S+L} protein during tumor progression (data not shown). However, since the antibody used for Bcl-X recognized both the short and long forms, immunohistochemical analyses would not determine whether one form increased and another form decreased during tumor progression. In addition, Western blotting or immunohistochemistry did not detect Bak (data not shown). These results suggest that Bax might be an important factor involved in the regulation of apoptosis during mammary tumor progression in this model system.

p53-independent Bax expression of mammary carcinomas

Although SV40-TAg protein would be expected to bind to and functionally inactivate p53, we wished to exclude further potential residual p53 activity which could be involved in regulating the expression of Bax in this model system. C3(1)/Tag transgenic mice carrying either *p53*^{+/+}, *p53*^{+/-} or *p53*^{-/-} alleles were generated (Maroulakou *et al.*, 1997) and analyzed for Bax expression. Western blotting demonstrated that Bax expression in carcinomas arising in mice carrying either the *p53*^{-/-} or *p53*^{+/-} alleles was comparable to those observed in carcinomas carrying wild-type *p53* (Figure 2F). These data demonstrate that in this transgenic model, a p53-independent pathway regulates Bax.

Effects of *bax* gene dosage on mammary tumor development in C3(1)/Tag transgenic mice

Survival rates and parameters of mammary tumor growth. In order to investigate the functional significance of *bax* during mammary tumor progression, mice carrying *bax* mutant alleles and the C3(1)/Tag gene were generated. C3(1)/Tag;*bax*^{+/+} and C3(1)/Tag;*bax*^{+/-} from the F₁ generation obtained by crossing C3(1)/Tag;*bax*^{+/-} with

Fig. 1. Apoptosis during mammary tumor progression in the C3(1)/TAG transgenic mice. (A and C) Nodular atypical hyperplasia. (B and D) Adenocarcinoma. Note many apoptotic cells are observed in the nodular atypical hyperplasia (C) but the levels are relatively decreased in the carcinoma (D). (E) Ultrastructure of apoptotic cells (arrow head) in atypical hyperplasia at 12 weeks of age. (F) Higher magnification of apoptotic cells in adenocarcinoma at 16 weeks of age. The nuclear fragments contain a characteristic half-moon of condensed chromatin (arrow heads). Cellular fragmentation results in the formation of apoptotic bodies, small membrane-bound vesicles containing nuclear fragments (arrows), and other disrupted organelles. (A and B) Hematoxylin and eosin (H&E), $\times 200$. (C and D) TUNEL, $\times 200$. (E and F) Transmission electron microscopy, $\times 4500$ (E), $\times 7000$ (F). (G and H) Sequential changes in levels of apoptosis (G) and PCNA immunoreactivity (H) during mammary tumor progression in the C3(1)/TAG transgenic mice. Normal (□), atypical hyperplasias (▨), nodular atypical hyperplasias (▩) and adenocarcinomas (■). Data presented are mean $\pm$ SD. Significantly different from values of normal epithelium at $P < 0.05$ (*) and $P < 0.01$ (**); significantly different from values of atypical hyperplasia at $P < 0.05$ (†) and $P < 0.01$ (‡). Fifteen C3(1)/TAG transgenic mice and nine non-transgenic FVB/N mice were examined.

Fig. 2. Expression of *bax*, Bcl-X_{SL} and p53 during mammary tumor progression in the C3(1)/TAg transgenic mice. (A) Northern blots analyses. Normal tissue (N), lanes 1 and 2; atypical hyperplasia (AH), lanes 3 and 4; and adenocarcinomas (Ca), lanes 5–8. *bax* α (1.0 kb) and β (1.5 kb) mRNA were markedly increased in atypical hyperplasias and adenocarcinomas. β-actin was used as an internal control. (B) Western blot analyses. Increased levels of Bax protein (21 kDa) was found in atypical hyperplasias (lanes 3–5) and carcinomas (lanes 6–8). Bcl-X_S (~20 kDa) was detected only in proliferative mammary lesions. Mouse thymus was used as a positive control (lane 9). (C–E) Immunohistochemistry of Bax during mammary tumor progression. Normal mammary epithelial cells exhibit weak cytoplasmic expression of Bax (C, ×400), whereas strong cytoplasmic expression is observed in atypical hyperplasias and nodular atypical hyperplasia (D, ×200), and adenocarcinoma cells (E, ×200). (F) Correlation of Bax, TAg and p53 expression in mammary carcinomas from the hybrid (FVB/N×129/Sv) C3(1)/TAg transgenic mice carrying different genotypes of *p53* by Western blotting. Overexpression of Bax is independent of the *p53* genotype. No changes in TAg expression were found among different *p53* genotypes (middle panel). Normal, lanes 1 (non-transgenic FVB) and 2 (non-transgenic Sv/129); carcinomas, lanes 3 and 4 (C3(1)/TAg;*p53*^{+/+}), lanes 5 and 6 (C3(1)/TAg;*p53*^{+/-}), lanes 7 and 8 (C3(1)/TAg;*p53*^{-/-}).

bax^{+/-} mice were used for analyses of survival and mammary tumor growth rates. Survival rates were significantly reduced in C3(1)/TAg;*bax*^{+/-} mice compared with C3(1)/TAg;*bax*^{+/+} mice (Figure 3A). Moreover, the incidence of mammary tumor development was significantly elevated in C3(1)/TAg;*bax*^{+/-} mice during 19–21 weeks of age compared with C3(1)/TAg;*bax*^{+/+} mice, although no differences of the tumor incidence were observed thereafter (Figure 3B). The multiplicity of mammary tumors was also higher in C3(1)/TAg;*bax*^{+/-} mice compared with C3(1)/TAg;*bax*^{+/+} mice after 19 weeks of age (Figure 3C), as was tumor volume (Figure 3D).

Histopathological analysis. C3(1)/TAg;*bax*^{+/+}, C3(1)/TAg;*bax*^{+/-} and C3(1)/TAg;*bax*^{-/-} transgenic mice from the F₂ generation were used for histopathological and molecular analyses. As shown in Figure 4A, at 20 weeks of age, the multiplicity of NAH was significantly increased in C3(1)/TAg;*bax*^{+/-} and C3(1)/TAg;*bax*^{-/-} mice compared with C3(1)/TAg;*bax*^{+/+} mice. The numbers of carcinomas tended to be increased in C3(1)/TAg;*bax*^{+/-} and C3(1)/TAg;*bax*^{-/-} mice (Figure 4B) compared with mice carrying *bax*^{+/+}. These differences were not due to changes in body weight as those were comparable between mice with the various *bax* genotypes (at 18 weeks of

Fig. 3. *bax* gene dosage effect on survival (A), incidence of palpable lesions (B), multiplicity of palpable lesions (C), and volume of the mammary tumors (D) in the hybrid (FVB/N×C57BL/6) C3(1)/TAG transgenic mice carrying mutant *bax* alleles. (A) Kaplan-Meier analysis of survival rates showed significant differences in survival between the *bax*^{+/+} and *bax*^{+/-} groups ($P < 0.01$). (B) The incidence of the mammary tumors was significantly increased in *bax*^{+/-} mice during 19–21 weeks of age compared with *bax*^{+/+} mice (* $P < 0.05$). (C) Multiplicity of the mammary tumors was much higher in *bax*^{+/-} mice than in *bax*^{+/+} mice. (D) Tumor volume tended to be larger in *bax*^{+/-} mice compared with *bax*^{+/+} mice. Data are presented as mean $\pm$ SD. Ten mice with *bax*^{+/+} genotype and 16 mice with *bax*^{+/-} genotype were examined. x-axis begins at 13 weeks of age (A–C).

age; *bax*^{+/+}, 24.6 ± 0.9 g; *bax*^{+/-}, 24.4 ± 2.5 g; *bax*^{-/-}, 24.1 ± 1.2 g).

Whole-mount analysis. Whole-mount preparation of the mammary glands demonstrated that the numbers of ductal lesions were much higher in C3(1)/TAG;*bax*^{+/-} and C3(1)/TAG;*bax*^{-/-} mice than in C3(1)/TAG;*bax*^{+/+} mice (Figure 4C–F). In order to determine whether the loss of wild-type *bax* alleles might affect normal mammary duct development and, therefore, indirectly affect the tumor phenotype, mammary gland development was assessed in mice carrying different *bax* genotypes but not the C3(1)/TAG transgene. Although the absence of one *bax* allele did not result in any obvious abnormality in general mammary ductal development, *bax*^{-/-} mice demonstrated thinner primary ducts and less branching of the tertiary and quaternary ducts than those observed in mice with the *bax*^{+/-} or *bax*^{+/+} genotypes (Figure 4G–I).

Expression of Bax protein. Bax protein levels in mammary glands containing multiple NAH lesions at 13 weeks of age were determined by Western blot analyses and correlated to the *bax* genotype of the transgenics: tissues from *bax*^{+/-} mice contained less Bax protein compared with that observed in *bax*^{+/+} mice, whereas no Bax protein was detectable in *bax*^{-/-} mice (Figure 5A). In the *bax*^{+/-} mice with accelerated tumor growth, Western blotting demonstrated that Bax expression was maintained in carcinoma cells (Figure 5B), although Bax expression was reduced compared with that in carcinomas with the *bax*^{+/+} genotype. In addition, genotyping of the mammary tumors which originated in the *bax*^{+/-} mice by PCR analysis confirmed that these tumors maintained the *bax*^{+/-} genotype, as both the wild-type and mutant *bax* alleles were identified in the carcinomas (Figure 5B). These data

strongly suggest that the accelerated tumor growth did not require the loss of function of the second *bax* allele, but rather was dose-dependent and could result from haploid insufficiency.

Apoptosis and cell proliferation. Levels of apoptosis as a function of the stages of mammary tumor progression (as assessed by the TUNEL assay) are presented in Figure 5C. Although levels of apoptosis among all *bax* genotypes were very similar in normal mammary ductal cells, the levels in AH and NAH were significantly lower in C3(1)/TAG;*bax*^{+/-} and C3(1)/TAG;*bax*^{-/-} mice compared with those in C3(1)/TAG;*bax*^{+/+} mice. Levels of apoptosis in invasive carcinomas, however, were not significantly different among the different *bax* genotypes. The percentage of PCNA-immunoreactive cells in normal mammary tissues and mammary lesions were not affected by *bax* gene dosage (Figure 5D).

Discussion

We have investigated potential molecular mechanisms that regulate apoptosis during critical stages of mammary tumor progression in the C3(1)/TAG transgenic mice. The marked increase in levels of apoptosis observed during the development of preneoplastic mammary lesions was associated with an elevation in the apoptosis-inducer Bax via a p53-independent mechanism. The loss of one or both alleles of *bax* in mice expressing the C3(1)/TAG transgene leads to the significant acceleration of mammary tumor development due to a reduced apoptotic response in the preneoplastic mammary lesions compared with those of C3(1)/TAG transgenic *bax*^{+/+} mice.

The molar ratio of Bax to other Bcl-2 family members

Fig. 4. Histological analyses for multiplicity of mammary preneoplastic lesions (nodular atypical hyperplasias) (**A**) and carcinomas (**B**) in the hybrid C3(1)/Tag transgenic mice with different *bax* genotypes. Numbers of the preneoplastic lesions were significantly increased in *bax*^{+/-} and *bax*^{-/-} mice than in *bax*^{+/+} mice (**P* < 0.05). Data presented as mean $\pm$ SD as described in Materials and methods. Numerals in parentheses indicate number of mice examined. (**C**–**I**) Whole mount of inguinal mammary glands demonstrates a much larger number of nodular lesions in mammary ducts in *bax*^{+/-} (**D**) and *bax*^{-/-} (**F**) mice compared with *bax*^{+/+} mice (**C** and **E**) at 16 and 20 weeks of age, respectively. (**G**–**I**) Evaluation of *bax* gene dosage on normal mammary duct development in mice carrying different *bax* genotypes without the C3(1)/Tag transgene. At 12 weeks of age, *bax*^{+/+} (**G**) and *bax*^{+/-} (**H**) mice show successive branching up to the fourth generation of ducts, while *bax*^{-/-} (**I**) mice show very thin ducts with less branching and poor development of terminal end buds. Carmine-aluminum potassium sulfate. Magnification, $\times 40$.

Fig. 5. (A) Bax expression in mammary preneoplastic lesions in the C3(1)/Tag transgenic mice carrying mutant *bax* alleles. Bax protein levels in mammary preneoplastic lesions of the *bax*^{+/-} double transgenics (lanes 5 and 6) show reduction in the Bax protein levels compared with *bax*^{+/+} mice (lanes 3 and 4), whereas no Bax protein was detectable in *bax*^{-/-} mice (lanes 7 and 8). There is no difference in the Bax protein levels in atypical hyperplasias (lane 1) and carcinomas (lane 2) from the parental C3(1)/Tag transgenic mice with *bax*^{+/+} in the FVB/N background compared with the C3(1)/Tag hybrid (FVB/N×C57BL/6) transgenics with *bax*^{+/+} (lanes 3 and 4). (B) Genotyping of tumors from different genetic backgrounds by PCR and correlation to Bax protein expression. Both wild-type and mutant *bax* alleles were detectable in tumors arising from *bax*^{+/-} mice (lanes 3 and 4) (upper panel). Bax expression was maintained in carcinoma cells with the *bax*^{+/-} genotype although reduced levels were observed compared with the *bax*^{+/+} genotype (lower panel). (C and D) Levels of apoptosis (C) and PCNA immunoreactivity (D) in mammary lesions of the double transgenics carrying different *bax* genotypes. (C) Significantly reduced apoptosis levels were observed in atypical hyperplasias and nodular atypical hyperplasias, but not carcinomas, from the *bax*^{+/-} and *bax*^{-/-} mice compared with that from *bax*^{+/+} mice (*P < 0.05, **P < 0.01). (D) Rates of PCNA immunoreactivity in mammary lesions were similar among all *bax* genotypes. Numbers in parentheses indicate number of mice examined.

appears to be critical in determining whether apoptosis is activated or suppressed. Bax has been shown to form homodimers as well as heterodimers with the apoptosis suppressors Bcl-2 (Oltvai *et al.*, 1993) or Bcl-X_L (Sedlak *et al.*, 1995). A predominance of Bax accelerates apoptosis in response to growth factor withdrawal, whereas a predominance of Bcl-2 greatly inhibits apoptosis (Oltvai

et al., 1993; Miyashita *et al.*, 1994). Alternative splicing of the *bax* gene generates three classes of transcripts (α , β and γ) of which the α mRNA has been shown to encode the 21 kDa protein which can heterodimerize with these proteins. In this transgenic model, both increased *bax* α and β mRNA were observed in the mammary lesions. In the present study, although no *bcl-2* mRNA and protein were detected at any stage of mammary tumor development, low levels of Bax expression were detected in normal mammary epithelium, with markedly elevated levels of Bax found in the preneoplastic and neoplastic lesions. Bax alone has been shown to increase apoptosis without interacting with other proteins such as Bcl-2 or Bcl-X_L (Gross *et al.*, 1998).

Levels of Bcl-X_L protein appeared to decrease slightly in the mammary preneoplastic and neoplastic lesions compared with normal epithelium. Previous reports have demonstrated that when excess Bcl-X_L is heterodimerized to Bax, apoptosis is inhibited (Yang *et al.*, 1995), whereas excess Bax in relation to Bcl-X_L activates apoptosis. In this transgenic system, it appears that excess Bax overwhelms the low amounts of Bcl-X_L and leads to apoptosis. The short form of Bcl-X, Bcl-X_S, also promotes apoptotic cell death and inhibits the function of Bcl-2 (Boise *et al.*, 1993). In general, Bcl-X_S protein is not expressed in normal mouse tissues but only in involuting mouse thymus (Krajewski *et al.*, 1994). In our studies, Bcl-X_S was detected only in the preneoplastic and neoplastic lesions. Thus, Bcl-X_S, in addition to Bax, could be an important factor regulating apoptosis during mammary tumor progression.

The functional significance of Bax expression during mammary tumor progression was confirmed *in vivo* using mice carrying the C3(1)/Tag transgene and mutant alleles for *bax*. Mice with mutant *bax* alleles had the expected reductions in Bax protein expression which was associated with an accelerated rate of mammary tumor development, increased tumor numbers, tumor volume and significantly reduced animal survival. The C3(1)/Tag mice with a *bax*^{+/-} or *bax*^{-/-} genotype showed significantly higher numbers of preneoplastic mammary lesions with decreased levels of apoptosis compared with transgenic mice with the *bax*^{+/+} genotype. However, the number of mammary lesions was much higher in the double transgenics carrying the *bax*^{+/-} genotype than that observed in the C3(1)/Tag; *bax*^{-/-} genotype. The *bax*^{-/-} mice exhibit mammary ductal hypoplasia with significantly less branching of the mammary ducts than mice with the *bax*^{+/-} or *bax*^{+/+} genotype, which is the likely reason for their reduced number of mammary lesions. It is possible that other systemic effects could influence mammary lesion development in the *bax*^{-/-} mice, such as the generalized lymphoid hyperplasia which occurs in the *bax*^{-/-} mice (Knudson *et al.*, 1995).

In order to determine whether accelerated tumor progression in *bax*^{+/-} mice was associated with or required the loss of the second wild-type *bax* allele, tumors arising in *bax*^{+/-} mice were genotyped for *bax* by PCR analyses. These results clearly demonstrated in multiple tumors that both the wild-type and mutant *bax* alleles were present. In addition, the level of expression of Bax protein in *bax*^{+/-} mammary carcinomas was ~50% of the Bax protein levels in *bax*^{+/+} tumors. Thus, haploid insufficiency of *bax* is able to significantly accelerate mammary tumor

progression in this model. Bax, therefore, appears to function in a dose-dependent manner in keeping with the stoichiometric theories of how apoptosis is modulated by the relative amounts of Bcl-2 family members. This implies that *bax* need not operate in the fashion of classical tumor-suppressor genes where the loss of both alleles is required for tumor development. In the case of *bax*, the loss of only one allele can have a significant impact on the acceleration of tumor development. Recently, haploid insufficiency of transforming growth factor β -1 has also been shown to modulate chemically-induced lung tumor development in transgenic mice (Tang *et al.*, 1998).

The loss of *bax* appeared to affect rates of apoptosis only at preneoplastic stages. Loss of *bax* was associated with a significant reduction in levels of apoptosis in AH and NAH lesions, while levels of apoptosis were not significantly different in carcinomas displaying different *bax* genotypes. These results suggest that although *bax* appears to suppress tumor growth by enhancing apoptosis, the Bax-protective effect is lost during the transition to invasive carcinomas. Since the rates of cellular proliferation for each stage of mammary tumor progression were similar for C3(1)/TAG mice regardless of the *bax* genotypes they carried, the acceleration of mammary tumor growth appears to be due primarily to the significantly decreased levels of Bax-induced apoptosis.

In our studies, we have not observed that *bax* is a target of mutation during tumor progression in these transgenic mice, as Bax expression seems to be maintained in the carcinomas. We have not excluded the possibilities that small mutations may disrupt Bax function or that mutations downstream of *bax* may alter Bax-induced apoptosis. Given the relatively rapid rate of progression from preneoplasia to carcinoma development, selective pressure for mutations in the Bax-apoptosis pathway may not be an important mechanism for tumor progression in this model. It is also possible that the activation of Bax protein is lost during the transition from preneoplasia to invasive carcinoma preventing the translocation of Bax from the cytosol to the mitochondrial membrane (Han *et al.*, 1998). We speculate that other potential mechanisms could lead to a reduced apoptotic response as lesions progress from preneoplasias to carcinomas.

The *p53* gene, a frequent target of mutation in humans (Greenblatt *et al.*, 1994), can induce apoptotic death of genetically damaged cells (Yonishi-Rouach, 1991). Although one pathway of *p53*-induced apoptosis may involve the upregulation of *bax*, *bax* is able to induce apoptosis in the presence of mutant *p53*, suggesting that Bax alone is capable of inducing apoptosis and functions downstream of *p53* (Han *et al.*, 1996). In fact, we observed high levels of Bax expression in C3(1)/TAG transgenic mammary carcinomas in mice carrying two *p53* null alleles. Our results clearly demonstrate that the regulation of Bax is not dependent upon a *p53*-mediated pathway in this model. A transgenic mouse model which develops choroid plexus tumors of the brain shows accelerated tumor growth with reduced apoptosis when those transgenic mice were crossed with *bax*-deficient mice (Yin *et al.*, 1997). However, in contrast to the results of this study, apoptosis in the choroid plexus tumor model appears to be *p53*-dependent. This suggests that the *p53* mechanism of Bax-induced apoptosis may be tissue specific.

In human breast cancer, reduced intensity of Bax immunostaining is associated with shorter overall survival, faster time to tumor progression and failure to respond to chemotherapy (Krajewski *et al.*, 1995). By reducing Bax expression in the transgenic model, tumor progression is accelerated and more aggressive. Although *bax* appears to be involved in the progression of both human and TAG-induced transgenic mammary cancers, the stage at which *bax* exerts its effect may differ between these species. In contrast, reduced Bax expression is not observed during progression of human prostate carcinoma (Krajewska *et al.*, 1996), suggesting that Bax expression and function in response to tumor development may be tissue specific.

In conclusion, our results demonstrate that *bax* is an important regulator of apoptosis and tumor progression during the preneoplastic stage of tumor development, but that genes other than *bax* are also important regulators of apoptosis, especially during the transition to invasive carcinoma.

Materials and methods

Generation of C3(1)/SV40 TAG transgenic mice carrying mutant *bax* or *p53* alleles

C3(1)/TAG transgenic mice have been described previously (Maroulakou *et al.*, 1994) and were maintained by breeding with wild-type FVB/N mice. Transgenic animals were identified by slot blot utilizing a ³²P-labeled SV40-specific probe hybridized to mouse tail DNA (Maroulakou *et al.*, 1994). Transgenic mice carrying null alleles of *p53* were generated by crossing the C3(1)/TAG^{+/−} transgenic mice with mice carrying a mutant *p53* allele (129/Sv background; Jackson Laboratories, Bar Harbor, ME) as previously described (Shibata *et al.*, 1996; Maroulakou *et al.*, 1997).

To generate transgenic mice carrying mutant *bax* alleles, C3(1)/TAG^{+/−} transgenic mice were bred with *bax*^{+/−}/C57BL/6 mice (Knudson *et al.*, 1995). Females from the F₁ generation carrying C3(1)/TAG^{+/−}; *bax*^{+/+} and C3(1)/TAG^{+/−}; *bax*^{+/−} were used for analyses of survival and tumor growth rates. An F₂ generation of C3(1)/TAG^{+/−} mice carrying either *bax*^{+/+}, *bax*^{+/−} or *bax*^{−/−} genotypes was generated by crossing C3(1)/TAG^{+/−}; *bax*^{+/−} mice with TAG-negative *bax*^{+/−} F₁ generation mice. Genotypes were identified by slot blot (SV40-TAG gene) and PCR analyses (*bax* gene) (Knudson *et al.*, 1995) using tail DNA. Females from the F₂ generation carrying *bax*^{+/+}, *bax*^{+/−} or *bax*^{−/−} were used for histopathological analysis. All manipulations of mice were performed in accordance with the guidelines of the Animal Care and Use Committee and in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, 1985), under an animal study proposal (NCI-FCRF-97-050) approved by the FCRDC Animal Care and Use Committee.

Individual body weight was recorded weekly. Mammary tumor size was measured weekly using a caliper and tumor volume was calculated using the following formula: the largest diameter × (the smallest diameter)² × 0.4 (Fueyo *et al.*, 1998).

Whole-mount preparations and histopathology

Transgenic and control mice were killed by CO₂ asphyxiation. Mammary gland whole-mount preparations were spread on a slide, fixed in 70% ethanol, rehydrated in distilled water, and stained with 0.2% carmine and 0.5% aluminum potassium sulfate. The whole mount was dehydrated in ethanol, cleared in xylene and mounted using Permount (Fisher Scientific, Fair Lawn, NJ). A portion of each mammary tissue sample was also immediately frozen on dry ice for subsequent molecular biological analyses, whereas the remaining mammary tissue was fixed in 4% paraformaldehyde or 10% phosphate-buffered formalin, embedded in paraffin, cut at a thickness of 4 μ m, and stained with H&E for histopathological examination. Histopathologic lesions were quantitated by summing the number of lesions in sections from two axillary and one inguinal mammary gland from each mouse. Glands from the same locations in all mice were used for these studies.

Electron microscopy

Mammary tissues from mice at 12 and 16 weeks of age were immediately fixed in 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer

(pH 7.2), post-fixed in 1% osmium tetroxide, dehydrated with increasing concentrations of ethanol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate followed by lead citrate. The sections were observed under a transmission electron microscope (model H7000 Hitachi, Tokyo, Japan) at 75 kV.

Immunohistochemistry

Sections were immersed in distilled water and heated by microwave for antigen retrieval and the avidin–biotin complex method was applied (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA). The following antibodies (Abs) were used in this study. Anti-bax rabbit polyclonal Ab (N-20, dilution 1:500) and anti-bcl-X_{S/L} rabbit polyclonal Ab (S-18, 1:20) from Santa Cruz Biotechnology, Santa Cruz, CA; anti-bak mouse monoclonal Ab (Ab-1, 1:50) and anti-p53 sheep polyclonal Ab (Ab-7, 1:500), which is wild-type and mutant p53-reactive, from Oncogene Science, Cambridge, MA; anti-PCNA mouse monoclonal Ab (PC-10, 1:400) from Dako, Carpinteria, CA; and anti-SV40-Tag mouse monoclonal Ab (Pab 101, 1:50) from PharMingen, San Diego, CA.

Apoptosis and cell proliferation

For quantitative analysis of apoptosis, 4-µm sections from paraffin-embedded mammary tissues were assayed by the TUNEL method (Apop Tag; Oncor, Gaithersburg, MD). Testes or thymus were used as positive controls. Since this technique may also stain cells undergoing necrotic cell death, areas of necrosis and regions immediately adjacent to necrotic areas within mammary carcinomas were excluded from the quantitation. The numbers of apoptotic cells per 1000 mammary ductal cells were counted in different lesions using an ocular micrometer disk (Fisher Scientific, Pittsburgh, PA); the numbers of cells counted in normal or early atypical lesions were occasionally <1000 cells. Levels of apoptosis were expressed as a percentage of total cells counted.

In order to evaluate cell proliferation, the numbers of PCNA-immunoreactive cells in late-G₁, S and G₂ were counted in mammary gland lesions and were expressed as a percentage of total cells counted.

Western blot

Whole-cell lysate (10 or 20 µg) from freshly isolated tissues was fractionated by 12 or 14% Tris-glycine gels under reducing conditions and transferred onto a PVDF membrane (Immobilon-P; Millipore, Bedford, MA). Primary antibodies used were as follows: anti-bcl-2 (N-19), bax (N-20) and bcl-X_{S/L} (S-18) from Santa Cruz Biotechnology; bak (Ab-1), SV40-TAG (Ab-2) and p53 (Ab-3) from Oncogene Science.

Northern blot

A 2.2 kb *EcoRI* fragment of the mouse *bcl-2* cDNA (clone# 22, kindly provided by Dr F. Mushinski, National Cancer Institute, Bethesda, MD), a 502 bp *EcoRI*–*BamHI* fragment of the mouse *bax* cDNA (kindly provided by Dr L. Hennighausen, National Cancer Institute, Bethesda, MD) were labeled with [α -³²P]dCTP by random oligonucleotide-primed synthesis. Total RNA (5 µg) was fractionated on a 1.0% formaldehyde agarose gel, transferred onto a nylon membrane and fixed by UV crosslinking. Membranes were hybridized with ³²P-labeled probes and washed using standard protocols. The membranes were then exposed to X-ray film at –70°C for varying periods of time.

Statistical analysis

The two-sided Student's *t*-test was used to determine whether significant differences existed between the mean values of the groups analyzed. Significant differences in lesion incidence were analyzed by Fisher's exact probability test and the Wilcoxon test was applied for the analysis of survival rates.

Acknowledgements

We thank Dr Anita B. Roberts for critical review of the manuscript and Dr Gilbert Smith for useful suggestions. We also thank Drs F. Mushinski and L. Hennighausen for kindly providing cDNA probes. We are grateful for the technical expertise of Lisa A. Birely and Darlene R. Reaver in the Animal Breeding and Carcinogenesis Section and the staff of the Pathology Histology Laboratory (Science Applications International Corporation, Frederick, MD) for preparation of the histology slides. The authors thank Jeff Nyswaner and Mario Anzano for assistance with genotyping the mice.

References

Boise, L., Gonzalez-Garcia, M., Postema, C., Ding, L., Lindsten, T., Turka, L., Mao, X., Nunez, G. and Thompson, C. (1993) bcl-X, bcl-2-

related gene that functions as a dominant regulator of apoptotic death. *Cell*, **74**, 597–608.

Chittenden, T., Harrington, E., O'Connor, R., Flemington, C., Lutz, R., Evan, G. and Guild, B. (1995) Induction of apoptosis by the Bcl-2 homologue Bak. *Nature*, **374**, 733–736.

El-Deiry, W.S. *et al.* (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Fueyo, J. *et al.* (1998) Overexpression of E2F-1 in glioma triggers apoptosis and suppresses tumor growth *in vitro* and *in vivo*. *Nature Med.*, **4**, 685–690.

Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C. (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855–4878.

Gross, A., Jockel, J., Wei, M.C. and Korsmeyer, S.J. (1998) Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. *EMBO J.*, **17**, 3878–3885.

Han, J., Sabbatini, P., Perez, D., Rao, L., Modha, D. and White, E. (1996) The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev.*, **10**, 461–477.

Han, J., Modha, D. and White, E. (1998) The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Oncogene*, **17**, 2993–3005.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) The p21 cdk-interacting protein Cip 1 is a potent inhibitor of G₁ cyclin-dependent kinases. *Cell*, **75**, 805–816.

Harris, J.R., Lippman, M.E., Veronesi, U. and Willett, W. (1992) Breast cancer. *N. Engl. J. Med.*, **327**, 390–398.

Hockenbery, D., Nunez, G., Millman, C., Schreiber, R. and Korsmeyer, S. (1990) Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature*, **348**, 334–336.

Kiefer, M., Brauer, M., Powers, V., Wu, J., Umansky, S., Tomei, L. and Barr, P. (1995) Modulation of apoptosis by the widely distributed Bcl-2 homologue Bak. *Nature*, **374**, 736–739.

Knudson, C., Tung, K., Tourtellotte, W., Brown, G. and Korsmeyer, S. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Nature*, **270**, 96–99.

Krajewska, M., Krajewski, S., Epstein, J., Shabaik, A., Sauvageot, J., Song, K., Kitada, S. and Reed, J. (1996) Immunohistochemical analysis of bcl-2, bax, bcl-X and mcl-1 expression in prostate cancers. *Am. J. Pathol.*, **148**, 1567–1576.

Krajewski, S., Krajewska, M., Shabaik, A., Wang, H.-G., Irie, S., Fong, L. and Reed, J. (1994) Immunohistochemical analysis of *in vivo* pattern of Bcl-X expression. *Cancer Res.*, **54**, 5501–5507.

Krajewski, S., Blomqvist, C., Franssila, K., Krajewska, M., Wasenius, V.-M., Niskanen, E. and Reed, J. (1995) Reduced expression of pro-apoptotic gene Bax is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. *Cancer Res.*, **55**, 4471–4478.

Maroulakou, I.G., Anver, M., Garrett, L. and Green, J.E. (1994) Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3 (1) simian virus 40 large tumor antigen fusion gene. *Proc. Natl Acad. Sci. USA*, **91**, 11236–11240.

Maroulakou, I.G., Shibata, M.-A., Jorcyk, C.L., Chen, X.-X. and Green, J.E. (1997) Reduced p53 dosage associated with mammary tumor metastasis in C3(1)/TAG transgenic mice. *Mol. Carcinogen.*, **20**, 168–174.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H., Lin, H., Liebermann, D., Hoffman, B. and Reed, J. (1994) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. *Oncogene*, **9**, 1799–1805.

Miyashita, T. and Reed, J. (1995) Tumor suppressor p53 is a direct transcription activator of the human bax gene. *Cell*, **80**, 293–299.

Oltvai, Z., Millman, C. and Korsmeyer, S. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609–619.

Sedlak, T., Oltvai, Z., Yang, E., Wang, K., Boise, L., Thompson, C. and Korsmeyer, S. (1995) Multiple Bcl-2 family members demonstrates selective dimerizations with Bax. *Proc. Natl Acad. Sci. USA*, **92**, 7834–7838.

Shibata, M.-A., Maroulakou, I.G., Jorcyk, C.L., Gold, L.G., Ward, J.M. and Green, J.E. (1996) p53-independent apoptosis during mammary tumor progression in C3(1)/SV40 large T antigen transgenic mice: Suppression of apoptosis during the transition from preneoplasia to carcinoma. *Cancer Res.*, **56**, 2998–3003.

- Tang,B., Bottinger,T., Jakowlew,S., Bagnail,K., Mariano,J., Anver,M., Letterio,J. and Wakefield,L. (1998) Transforming growth factor- β 1 is a new form of tumor suppressor with true haploid insufficiency. *Nature Med.*, **4**, 802–807.
- White,E. (1996) Life, death and the pursuit of apoptosis. *Genes Dev.*, **10**, 1–15.
- Yang,E., Zha,J., Jockel,J., Boise,L., Thompson,J. and Korsmeyer,S. (1995) Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell*, **80**, 285–291.
- Yin,C., Knudson,C., Korsmeyer,S. and Dyke,T. (1997) Bax suppresses tumorigenesis and stimulates apoptosis *in vivo*. *Nature*, **385**, 637–640.
- Yin,X.-M., Oltvai,Z. and Korsmeyer,S. (1994) BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature*, **369**, 321–323.
- Yonishi-Rouach,E. (1991) Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature*, **352**, 345–347.

Received January 27, 1999; revised and accepted March 22, 1999