[CANCER RESEARCH 62, 3373–3376, June 15, 2002]

Advances in Brief

Critical Requirement of BAX for Manifestation of Apoptosis Induced by Multiple Stimuli in Human Epithelial Cancer Cells[1]

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Abstract

Studies with mouse embryo fibroblasts deficient for the BCL-2 family multidomain proapoptotic proteins BAX and BAK have revealed that both of these proteins are essential for apoptosis induced by multiple stimuli, suggesting that these proapoptotic proteins are functionally overlapping in these cells [M. C. Wei *et al.*, Science (Wash. DC), *292:* 727–730, 2001; W. X. Zong *et al.*, Genes Dev., *15:* 1481–1486, 2001]. We have determined the effect of several different apoptotic stimuli in a *Bax*-deficient human epithelial cancer cell line (HCT116BaxKO). We show that this cell line expresses functional BAK protein and is defective in manifestation of apoptosis induced by the BH3-only proteins BIK and BID as well as extrinsic stimuli that engage the death receptors, tumor necrosis factor receptor, tumor necrosis factor-related apoptosis-inducing ligand receptor, and Fas. In addition, this cell line is deficient for apoptosis induced by cytotoxic agents such as UV, staurosporine, and thapsigargin that induce either mitochondrial or endoplasmic reticulum stress. Our results suggest that BAX plays a critical role in the manifestation of apoptosis paradigms induced by multiple stimuli in human epithelial cancer cells. Our results also suggest that the integrity of BAX may have important consequences in the progression of epithelial tumors and in determining the outcome of chemotherapeutic regimens of such tumors.

Introduction

The BCL-2 family proteins play a central role in apoptosis regulation (1) and appear to modulate oncogenesis (2). Several cellular and viral BCL-2 family proteins such as BCL-2, BCL-xL, and EBV-BHRF1 suppress apoptosis, whereas a number of other cellular BCL-2 family proteins promote apoptosis. The antiapoptotic BCL-2 family proteins are generally characterized by the presence of four conserved BH[3] domains (BH1–4). The BCL-2 family proapoptotic proteins fall into two classes. Proteins such as BAX and BAK share more extensive homology with BCL-2. These proteins designated as "multidomain" BCL-2 family proapoptotic proteins share homology over three domains (BH1, BH2, and BH3) with BCL-2 and lack the fourth domain (BH4), which is unique to the antiapoptotic proteins. In contrast, several other proapoptotic proteins share only a single domain (BH3) with BCL-2 and are designated as the "BH3-only" proteins. The BH3-only proteins serve as effectors of apoptotic signaling and connect the various apoptotic stimuli with core apoptotic machinery (reviewed in Ref. 3). The expression of BH3 domain-only proapoptotic proteins are activated either transcriptionally or posttranscriptionally (reviewed in Ref. 4).

Recent studies using nullizygous MEFs deficient in the multidomain proapoptotic proteins BAX and BAK suggest that these proteins are constituents of the core apoptosis machinery in animal cells, and they lie downstream of the BH3-only proteins. Both BAX and BAK appear to exist in inactive conformations and are "activated" in response to various apoptotic stimuli (5, 6). The inactive form of BAX is present in the cytosol as a monomer (7) and is translocated to the outer mitochondrial membrane after activation, where it appears to form oligomeric complexes (6). Both inactive and active forms of BAK are located on the outer mitochondrial membrane and upon activation form multimeric complexes (8). MEFs deficient in both BAX and BAK have been shown to be refractory for apoptosis induced by various agents that induce either mitochondrial stress or endoplasmic reticulum stress (9). These cells are also resistant to apoptosis induced by ectopic expression of various BH3-only proteins such as BID, BIM, and NOXA (9–11). MEFs singly deficient for either BAX or BAK were not significantly defective for apoptosis induced by the various agents (9–11). These studies have led to the suggestion that both BAX and BAK constitute critical components of mammalian apoptosis machinery, and these multidomain proteins may be functionally overlapping (9–11). However, it remains to be established if the functional redundancy of BAX and BAK observed in MEFs is also relevant to cells of different lineage and from different species.

Zhang *et al.* (12) have created a *Bax*-deficient human colorectal cell line and showed that these epithelial cells are resistant to apoptosis by chemopreventive nonsteroidal anti-inflammatory drugs such as sulindac or indomethacin. While surveying various human cancer cell lines for their apoptotic response to the BH3-only protein BIK (13), we discovered that the human *Bax*-knockout cell line (HCT116 BaxKO) derived from the colorectal cancer cell line HCT116 was strongly resistant to BIK-induced apoptosis. This prompted us to examine the effect of several well-known apoptotic agents that induce apoptosis attributable to mitochondrial or endoplasmic reticulum stress. Here, we report that this human *Bax* knockout epithelial cell line is deficient in manifestation of the apoptosis response when exposed to multiple apoptotic stimuli such as ectopic expression of the BH3-only proproteins, extrinsic stimuli such as ligands that engage "death" receptors, as well as other agents that induce mitochondrial or endoplasmic reticulum stress. These results suggest that the multidomain proapoptotic proteins may not have fully overlapping functions in all cell types and that BAX may play a more important role in the apoptosis paradigms in human epithelial cancer cells.

Materials and Methods

Cell Lines and Transfection. The HCT116-derived human colon carcinoma cells lines HCT116Bax and HCT116BaxKO have been described elsewhere (12) and were a gift from Dr. B. Vogelstein. HCT116 Bax is a subclone of HCT116 that is heterozygous for the *Bax* gene and expresses BAX protein. BaxKO is a mutant in which the remaining *Bax* allele has been disrupted by homologous recombination and does not express any BAX protein. HCT116Bax and HCTBaxKO cells were grown in McCoy's 5A medium supplemented with 10% FCS. To reintroduce BAX expression back into

Received 3/8/02; accepted 4/25/02.

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[1] This study was supported by research Grants CA-73803 and CA-33616.

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[3] The abbreviations used are: BH, BCL-2 homology domain; MEF, mouse embryo fibroblast; TNF, tumor necrosis factor; TNF-R, TNF receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Ad, adenovirus; β-gal, β-galactosidase.

BaxKO cells, pcDNA3-BAX was transfected into BaxKO cells with FuGene6 transfection reagent (Roche). The transfected cells were selected with 600 μg/ml G418. Individual colonies were picked and screened for BAX protein expression and apoptotic response.

Cell Death Assays. Cells were plated at 5×10^5 cells/dish in 35-mm dishes and treated on the following day with staurosporine (0.05 mM) for 48 h, thapsigargin (0.5 μM) for 48 h, human TNF-α (1 ng/ml) plus cycloheximide (25 μg/ml), or monoclonal anti-Fas antibody (250 ng/ml) plus cycloheximide (25 μg/ml) for 24 h or exposed to UV radiation (254 nm 65 W germicidal lamp at a distance of 33 cm for 120 s) and assayed 24 h later. Total cells (floating and attached) were harvested by trypsinization, washed with PBS, and assayed for Annexin-V binding and 7-amino-actinomycin D (7-AAD) uptake by flow cytometry on a GuavaPC personal flow cytometer (Guava Technologies). For transient transfection assays, cells were plated at 5×10^5 cells/dish in 35-mm dishes and transfected the following day with 1 μg of pcDNA3 BIK or BID or empty vector plus 0.25 μg β-gal plasmid and fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 24 h later. Round blue apoptotic and total blue cells were counted to determine the percentage of apoptotic cells.

Cloning of Bak cDNA from BaxKO Cells. cDNA of the *Bak* gene was made from cytoplasmic RNA isolated from BaxKO cells using the Cells-to-cDNA kit (Ambion) and *Bak*-specific primer CCGCTCGAGTCATGATTTGAAGAATCTTCG. The *Bak* cDNA was amplified using the following primers: GGAATTCGGCTTCGGGGCAAGGCCCAGGT and CCGCTCGAGTCATGATTTGAAGAATCTTCG and Vent DNA polymerase (New England Biolabs). The PCR product was gel purified, cut with *Xho*I, and cloned in-frame with the HA tag of pcDNA3-HA (Blunt ended *Bam*HI to *Xho*I).

Immunoblot Analysis. Immunoblots were performed using standard protocols. In brief, whole cell extracts were prepared in lysis buffer containing 50 mM Tris (pH 8), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with protease inhibitor mixture (Roche Molecular Biochemicals, Mannheim, Germany). Cells were lysed for 1 h on ice. The lysates were clarified by centrifugation at 13,000 rpm for 15 min at 4°C. Supernatants were collected, and the amount of soluble proteins was then quantified by detergent-compatible protein assay (Bio-Rad, Richmond, CA). An equal amount of total protein (50 μg) was resolved in 15% SDS-PAGE, followed by blotting onto nitrocellulose membrane. Membranes were probed with anti-Bax NT (Upstate Biotechnology, Lake Placid, NY) and anti-Bak NT (Upstate Biotechnology) rabbit polyclonal antibodies. Horseradish peroxidase-conjugated goat antirabbit IgG was used as secondary antibody. Protein bands were visualized by ECL Western blotting detection reagent (Amersham), according to the manufacturer's protocol.

Results and Discussion

Expression of BAK in HCT116Bax and BaxKO Cells. Because the multidomain proapoptotic proteins BAX and BAK appear to be functionally overlapping in MEF, it is important to know the status of BAK expression in HCT116Bax (*Bax* +/−) and HCT116BaxKO (*Bax* −/−) cells. Western blot analysis (Fig. 1*A*) revealed that HCT116Bax and the BaxKO cells expressed significant levels of BAK, and the level of expression was comparable with that of another human epithelial cancer cell line, MCF-7. A similar analysis of BAX expression indicated that there was no detectable level of BAX expression in HCT116BaxKO cells, whereas the level of BAX expression in HCT116Bax cells was similar to that of MCF-7 (Fig. 1*A*). To determine whether the BaxKO cells express the functional *Bak* gene, we cloned the *Bak* cDNA from the mRNA prepared from these cells and determined the proapoptotic activity of the cDNA clones. Transient transfection of four different *Bak* cDNA clones in MCF-7 cells induced similar levels of apoptosis as the *wt Bak* cDNA (Fig. 1*B*). DNA sequence analysis of the cDNA clones indicated that three clones were identical to the *wt Bak*, whereas the fourth clone (clone 3) contained 1-bp change, resulting in a 1-amino acid substitution at position 14 (Cys→Arg). It appears that this substitution does not affect the proapoptotic activity of BAK. It is likely that the 1-bp change in one of the cDNA clones might be the result of PCR amplification because partial sequence analysis of six other clones did not reveal the presence of a similar mutation. These results suggest that HCT116BaxKO cells express functional BAK protein.

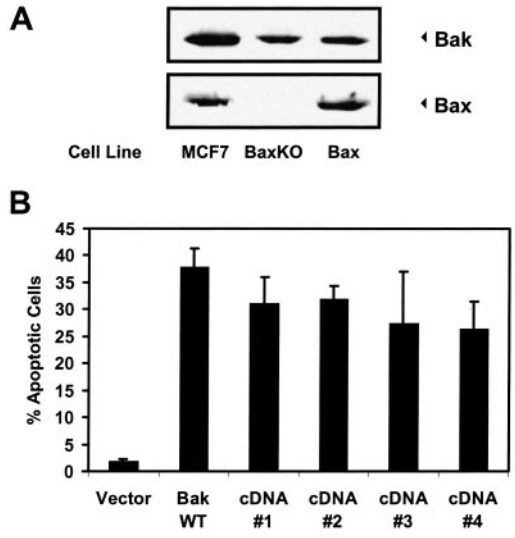

Fig. 1. Expression of BAK and BAX in HCT116 cells. *A*, immunoblot analysis of BAX and BAK in HCT116Bax (Bax+/−), HCT116BaxKO (Bax−/−), and MCF-7 cells. *B*, transient apoptosis assay of *Bak* cDNA clones. The various cDNA constructs were transfected in MCF-7 cells along with the β-gal reporter construct and the percentage of apoptotic cells among the transfected cells were quantified. *Bars*, SD.

Effect of BH3-Only Proapoptotic Proteins. The role of BAX on apoptosis induced by two BH3-only proapoptotic proteins BIK and BID was examined in cells transiently transfected with vectors that express these proteins. HCT116Bax and BaxKO cells were transfected with pcDNA3-BIK or pcDNA3-BID, along with the β-gal reporter plasmid and the apoptotic cells, were quantified by microscopic examination of β-gal-expressing cells. Both BIK and BID induced significant apoptosis in HCT116Bax cells, whereas no detectable apoptosis was observed in BaxKO cells (Fig. 2*A*). We note that the level of apoptosis seen in HCT116Bax cells transfected with BID is lower than that observed in cells transfected with BIK. This might be the result of transfection of full-length BIK rather than NH_2-terminally truncated tBID. The effect of BIK was further studied using an adenovirus vector (Ad-BIK) that expresses BIK (14). The cells were either mock infected or infected with Ad-BIK, and apoptosis was quantified by counting Annexin-V-positive cells at 24 h after infection. HCT116Bax cells infected with Ad-BIK exhibited significant apoptosis when compared with Ad-BIK-infected HCT116BaxKO cells (Fig. 2*B*). We believe that the estimation based on Annexin-V staining may be an underestimate. Microscopic examination of HCT116Bax cells infected with Ad-BIK exhibited massive apoptotic morphology, whereas BaxKO cells infected with Ad-BIK resembled the mock-infected cells without significant apoptotic morphology. These results suggest that the BaxKO cells are deficient in manifestation of apoptosis mediated by the BH3-only proteins BIK and BID.

Apoptotic Response to Multiple Stimuli. To determine whether the BAX-deficient cells are also refractory to apoptosis induced by other stimuli, we examined the effect of ligands that engage the various death receptors, TNF-R, TRAIL receptor, and Fas. HCT116Bax and HCTBaxKO cells were treated with TFN-α or TRAIL or with the anti-Fas antibody, and the apoptotic response was measured on the basis Annexin-V staining (Fig. 3). These results suggest that the BaxKO cells exhibit significant resistance to all three death receptor ligands, compared with the parental cell line. It appears that treatment of the BaxKO cells with TRAIL at concentrations that kill most of the BAX-expressing cells does not significantly affect cell

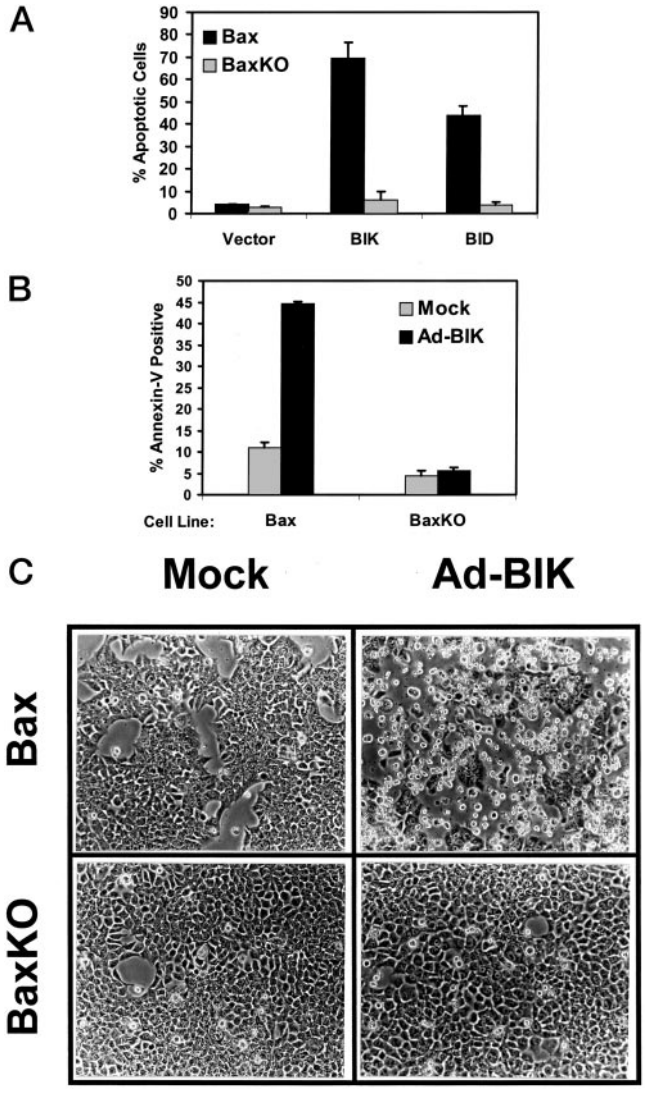

Fig. 2. Requirement of BAX for BIK- and BID-induced apoptosis. *A,* transient apoptosis assay of BIK and BID. HCT116Bax and HCT116BaxKO cells were transfected with the empty vector or vectors that express BIK or BID, and apoptotic cells were quantified. *B,* effect of adenovirus BIK recombinant. Cells were either mock infected or infected with Ad-BIK at an multiplicity of infection of 50 plaque-forming units/cell, and apoptosis was measured by Annexin-V staining. *Bars,* SD. *C,* photomicrographs of cells infected with Ad-BIK. Phase contrast micrographs were taken at a magnification of ×100.

proliferation in short-term growth assays (not shown). During the preparation and review of the manuscript, other groups also reported that *Bax* is essential for TRAIL-induced apoptosis (15–17). In addition to the ligands of the death receptors, we have also examined the effects of various cytotoxic agents that cause stress in the endoplasmic reticulum (thapsigargin) or in mitochondria (UV and staurosporine). Here also the BaxKO cells exhibited reduced apoptotic response when treated with these agents (Fig. 3).

Apoptotic Response in Cells with Reintroduced BAX. To ascertain whether HCT116BaxKO cells are refractory to apoptosis because of the absence of BAX, we introduced the *Bax* cDNA into these cells by transfection. Five different clones were isolated from the transfected cells, and three were found to stably express BAX over an extended period of several months in culture. Two cell clones that express different levels BAX (Fig. 4*A*) were used to determine their apoptotic response to treatment with TNF-α (Fig. 4*B*). Both clones manifested apoptotic response to TNF-α, and the level of response was similar to that of the HCT116Bax cells. The cells that ectopically express BAX were also tested for their response to infection with Ad-BIK. Both clones exhibited significant apoptotic response like HCT116Bax cells (Fig. 4*C*). These results suggest that the integrity of BAX is essential for manifestation of apoptosis induced by different stimuli. It is interesting that the relative level of BAX expression in these cells did not significantly influence the level of apoptosis.

We have shown that the apoptosis paradigms mediated by two different BH3-only proapoptotic proteins, BIK and BID, require the multidomain proapoptotic protein BAX in epithelial human cancer cells to induce apoptosis. Furthermore, in these cells BAX is also essential for apoptosis induced by extrinsic stimuli that engage the death receptors such as TNF-R, TRAIL receptor, and Fas as well as other cytotoxic agents that cause mitochondrial or endoplasmic reticulum stress. Previous studies, with fibroblasts from mouse embryos doubly deficient for both BAX and BAK, have indicated that they are defective for apoptosis mediated by several BH3-only proteins and various other apoptotic stimuli (9, 10). MEFs singly deficient for either BAX or BAK appear to exhibit normal apoptotic response to these agents, suggesting that both BAX and BAK are functionally overlapping, and both these multidomain proapoptotic constitute the "gateway" for apoptosis. Our results further strengthen the view on the requirement of a multidomain proapoptotic protein for manifestation of apoptosis in mammalian cells. Although our results do not

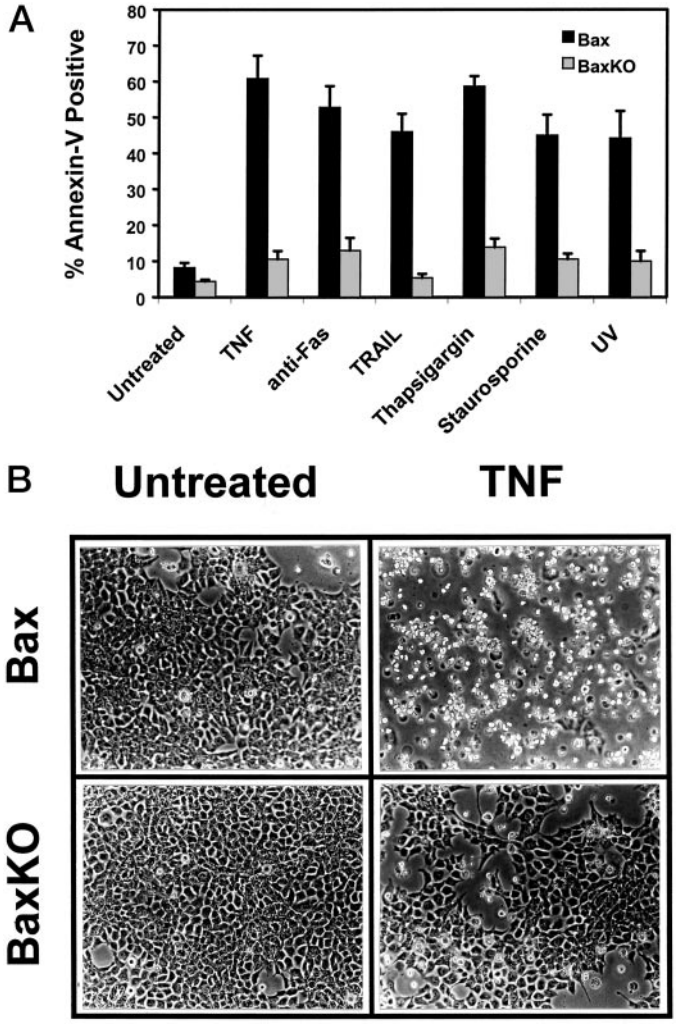

Fig. 3. Effect of multiple apoptotic stimuli. *A,* Cells were treated with TNF-α, TRAIL, anti-Fas antibody, thapsigargin, staurosporine, or UV and Annexin-V-positive cells were quantified. *B,* photomicrographs of cells treated with TNF-α. *Bars,* SD.

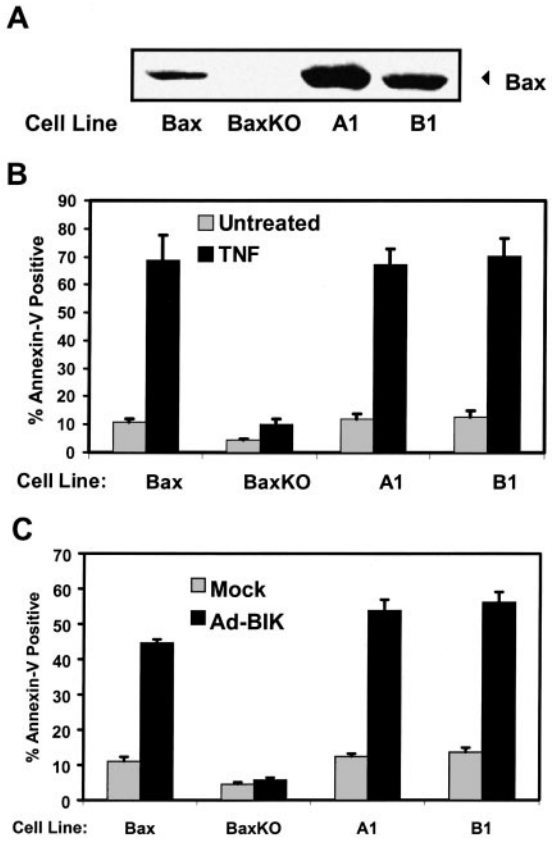

Fig. 4. Effect of ectopic BAX expression. HCT116BaxKO cells were transfected with the BAX expression vector, and two clones (A1 and B1) that express different levels of BAX (*A*) were used to determine their sensitivity to TNF-α (*B*) or infection with Ad-BIK (*C*) as in Figs. 2 and 3. *Bars*, SD.

exclude any potential role for BAK, they suggest that BAX may play a more critical role in the apoptotic response of human epithelial cancer cells. These results raise the possibility that the requirement of a multidomain proapoptotic protein may be cell type dependent. In a previous report, a requirement of BAX for apoptosis induced by the BH3-only proteins HRK and BIM in primary neurons from Bax−/− mice has been observed (18), suggesting a more important role for BAX in nonfibroblast cells.

The observation that BAX constitutes a critical checkpoint for the apoptosis paradigms in epithelial cells is of much importance with regard to oncogenesis and cancer therapy. Most human malignancies are of epithelial origin. The human *Bax* gene is prone to mutational inactivation because of the presence of a simple (G)8 repeat. More than half of the gastrointestinal cancers of the microsatellite mutator phenotype contain frameshift mutations in the G(8) repeat of the *Bax* gene (19, 20). Mutations in the *Bax* gene have been observed in other malignancies as well (20). Mutational inactivation of *Bax* has also been implicated in conferring survival advantage during clonal evolution of tumors of microsatellite mutator phenotype (21, 22). It is possible the tumor cells containing mutations in the *Bax* gene may be resistant to harsh apoptosis-inducing conditions during tumor expansion. In light of the previous results by from the Volgelstein group (12) and the present study, it appears that BAX may play a dominant role in conferring chemosensitivity to epithelial tumor cells. Although the BaxKO cell line is positive for p53, it is resistant to UV, suggesting that the *Bax*-deficient cells might be resistant to other DNA-damaging agents, such as chemotherapeutic drugs in epithelial cancer cells. Similarly, the integrity of the *Bax* gene would be important for successful chemotherapeutic regimens based on the cytokine TRAIL.

It is now well established that BAX is an essential component of the mitochondrial apoptosis program. The BH3-only proteins and other apoptotic stimuli appear to activate BAX by unknown mechanism(s) to cause mitochondrial dysfunction and release of various apoptotic factors. The critical requirement of BAX for manifestation of apoptosis induced by multiple stimuli may provide a system for the genetic analysis of BAX activation in human cells.

Acknowledgments

We thank B. Vogelstein for the kind gifts of HCT116Bax and BaxKO cell lines.

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