Overexpression of the Death-promoting Gene bax-α Which Is Downregulated in Breast Cancer Restores Sensitivity to Different Apoptotic Stimuli and Reduces Tumor Growth in SCID Mice

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Abstract

We have studied the expression of members of the bcl-2 family in human breast cancer. The expression pattern of these genes in breast cancer tissue samples was compared with the expression pattern in normal breast epithelium. No marked difference with regard to bcl-2 and bcl-xL expression was observed between normal breast epithelium and cancer tissue. In contrast, bax-α, a splice variant of bax, which promotes apoptosis, is expressed in high amounts in normal breast epithelium, whereas only weak or no expression could be detected in 39 out of 40 cancer tissue samples examined so far. Of interest, downregulation of bax-α was found in different histological subtypes. Furthermore, we transfected bax-α into breast cancer cell lines under the control of a tetracycline-dependent expression system. We were able to demonstrate for the first time that induction of bax expression in breast cancer cell lines restores sensitivity towards both serum starvation and APO-I/Fas–triggered apoptosis and significantly reduces tumor growth in SCID mice. Therefore, we propose that dysregulation of apoptosis might contribute to the pathogenesis of breast cancer at least in part due to an imbalance between members of the bcl-2 gene family. (*J. Clin. Invest.* 1996. 97:2651–2659.) Key words: APO-I/Fas • bcl-2 family • epithelium • mammary gland • malignancies

Introduction

Apoptosis or programmed cell death plays an important role in the regulation of tissue development, differentiation, and homeostasis (1). Therefore, it is possible that dysregulation of apoptosis contributes to the pathogenesis of cancer. This has been shown in the case of malignant lymphomas where resistance towards apoptosis is an important factor for tumor development (2–6). Apoptosis can be distinguished biochemically and morphologically from necrosis by the following criteria: (*a*) chromatin condensation, (*b*) membrane blebbing, (*c*) the appearance of apoptotic bodies, and (*d*) fragmentation of the genomic DNA (1).

The role of apoptosis has been studied extensively in the neuronal and lymphoid system during the past few years. It is well documented that apoptosis occurs during antigen-mediated selection processes within B and T cell ontogeny (7). In epithelia, apoptotic mechanisms are not as precisely understood as in the lymphoid system, although it has been reported that apoptosis is involved in the regulation of epithelial development and differentiation. For example, in hormone-dependent tissues like mammary and prostate gland, apoptosis is readily induced by hormone ablation and by treatment with antiestrogens or antiandrogens (8, 9). Programmed cell death is also observed during involution of the lactating breast (10, 11). Onset of cell death in these epithelia is accompanied by the induction of multiple genes, e.g., *TRMP-2*, *stromelysin*, and *c-fos/jun D* (9, 11, 12). Although little is known about the mechanisms which regulate apoptosis in epithelia it is conceivable, however, that defects in apoptosis-regulating genes are involved in the pathogenesis of human carcinomas. This hypothesis is supported by the fact that the tumor suppressor gene product p53, which is frequently mutated or deleted in different neoplasias including breast cancer, is involved in regulating apoptosis (13–17).

Apart from these genes, a well characterized molecule involved in apoptosis is the APO-1/Fas (CD95) antigen (18, 19). The CD95 antigen belongs to the TNF/NGF receptor superfamily (20, 21) and agonistic antibodies directed against this antigen have been shown to induce apoptosis in normal and malignant hematopoietic cells (18, 19, 22, 23). Recently, the CD95 ligand has been cloned and was shown to induce apoptosis. CD95 expression has also been observed in a wide variety of epithelial tissues (24). In the normal mammary gland CD95 is easily detected by immunohistochemistry on the acinar and ductular epithelial cells. In breast carcinoma most tumors are CD95 positive (24).

Another important regulator of apoptosis is the bcl-2 oncogene which was identified at t(14;18) chromosomal translocation breakpoints in the majority of follicular B cell lymphomas (4). These translocations lead to juxtaposition of the bcl-2 gene on chromosome 18 with the immunoglobulin heavy chain gene (IgH) on chromosome 14 (5). Subsequent overexpression of the bcl-2 gene renders the lymphoma cells resistant to apoptosis. Thus, dysregulation of apoptosis may be a causative event in the evolution of B cell malignancies. In addition to lymphoid cells the bcl-2 gene is also expressed in other tissues, such as mammary gland, prostate, complex differentiating epithelia with long lived stem cells (intestine, skin), and fully dif-

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ferentiated noncycling cells like neurons (25). However, not all long-lived cell types express the bcl-2 gene. In muscle cells and in other tissues of mesodermal origin bcl-2 is not expressed. Thus, bcl-2 gene expression seems to be restricted to tissues which are able to undergo apoptosis.

Recently, two bcl-2–related genes were identified: bcl-x and bax, both revealing significant sequence homology to bcl-2 (26, 27). Differential splicing of the bcl-x mRNA gives rise to a short (bcl-xs) and to a long (bcl-xL) splice variant with different functions: bcl-xL has an antiapoptotic effect whereas bcl-xs suppresses bcl-2 function. In vivo, the long splice variant bcl-xL is predominantly expressed in postmitotic quiescent cells, whereas bcl-xs is expressed in tissues with a high cell turnover. bax mRNA is also differentially spliced leading to bax-α with a length of 1 kb, bax-β with a length of 1.5 kb, and bax-γ (27). bax-γ lacks the small exon 2 (53 bp) resulting in a γ form of bax-α or bax-β. Recently, another splice variant of bax has been cloned, bax-δ, lacking exon 3 (28). The function of the β and δ variants as well as the two γ forms is still unclear. The bax-α mRNA encodes a bax protein that promotes apoptosis due to its ability to form heterodimers with bcl-2 (27). Thus, bcl-2, bcl-x, and bax are closely related factors which regulate apoptosis.

It has not yet been determined whether bcl-2 or bcl-2 related genes play any role in the development of breast cancer. It was reported that the bcl-2 gene is expressed in breast cancer specimens. However, similar levels of expression were identified in normal tissues (29, 30). In other cases loss of bcl-2 gene expression was somewhat linked to markers of poor prognosis (29). Others have reported that the predictive role of bcl-2 expression in lymph node–negative patients was mainly dependent on p53 expression (31).

Recently, we have found that bax-α mRNA is expressed in high amounts in normal breast tissue, whereas only weak or no expression could be detected in breast cancer cell lines and in 10 different breast cancer tissue samples, which were all of the ductal invasive type (32). Furthermore, we showed that in contrast to breast cancer–derived cell lines, which expressed low levels of bax-α, normal epithelial cell lines displaying high amounts of bax-α were highly sensitive to induction of programmed cell death by both serum starvation and APO-1/Fas (CD95) triggering. Therefore, in this study we were interested whether our preliminary data on mRNA level can be confirmed by immunohistological and Northern blot analysis of a larger number of histologically different breast cancer samples. To analyze the role of the bcl-2 gene family and of the APO-1/Fas system in regulating apoptosis in epithelial cells and to define its potential function in the development of breast cancer we transfected bax-α into breast cancer cell lines. We were interested in whether induction of bax-α expression could restore sensitivity of serum starvation or APO-1/Fas–mediated apoptosis in breast cancer cells and if so, whether this has consequences for tumor growth in vivo.

Methods

Cells and tissues. The following human cell lines were analyzed in this study. The human breast cancer cell lines MCF-7 (estrogen receptor [ER][1] positive) and R30C (ER negative) (a generous gift from Prof. Scherneck and Dr. Theile, Berlin, Germany). Breast cancer samples and breast tissue samples from patients with fibrocystic disease were collected at surgery and processed as described previously (33). Tumor-free (normal) breast tissue as determined by histology was obtained from the same patients.

Culture conditions and reagents. Cells were maintained in RPMI 1640 (Seromed-Biochrom, Hamburg, Germany), 10% heat-inactivated FCS, 2 mM L-glutamine (Gibco, Karlsruhe, Germany), and penicillin-streptomycin (Seromed-Biochrom). Where indicated, cells were cultured in the presence of tetracycline at a final concentration of 2 μg/ml.

[^{3}H]Thymidine incorporation assay. 5×10^4 cells/well were cultured in medium in 96-well round-bottom plates at 37°C for 48 h. Where indicated, cells were cultured with culture medium in the presence of 1 μg/ml mAbs FII23c (22) or anti–APO-1 (22). The cells were pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Braunschweig, Germany) after 2 d of culture and DNA synthesis was measured during the last 16 h of culture. The cells were harvested onto glass filters and the incorporated radioactivity was measured using a TopCount counter (Canberra Packard, Frankfurt, Germany).

Determination of apoptotic cells by acridine orange staining. After serum depletion cultures were seeded at 3×10^5 cells per 1 ml tissue culture well in RPMI 1640, stained with acridine orange (5 μg/ml), and observed by fluorescence microscopy. The number of fragmented nuclei, which reliably indicate apoptosis, was determined.

DNA fragmentation assay. For DNA fragmentation analysis, cell lysates were obtained by incubating a cell pellet containing 10^6 cells in 20 ml lysis buffer (10 mM EDTA, 50 mM Tris, pH 8, 0.5% sarcosyl, 0.5 mg/ml proteinase K) for 1 h at 50°C. After addition of 5 ml of RNase (1 mg/ml) and another incubation for 1 h at 50°C, lysates were electrophoresed in a 2% agarose gel containing ethidium bromide. The gel was run in a solution containing 26 mM Na_2HPO_4, 33 mM NaH_2PO_4, 10 mM EDTA, and photographed under ultraviolet illumination.

Stable breast cancer cell lines R30C and MCF-7 expressing inducible bax-α. In the first step the plasmid pUHD 15-1 (34) containing the tetracycline repressor gene fused with the viral VP16 coding region was linearized with ScaI, cotransfected with the resistance plasmid pUC18 (puromycin resistance) (Boehringer Mannheim, Mannheim, Germany), and selected for stable R30C and MCF-7 cell lines.

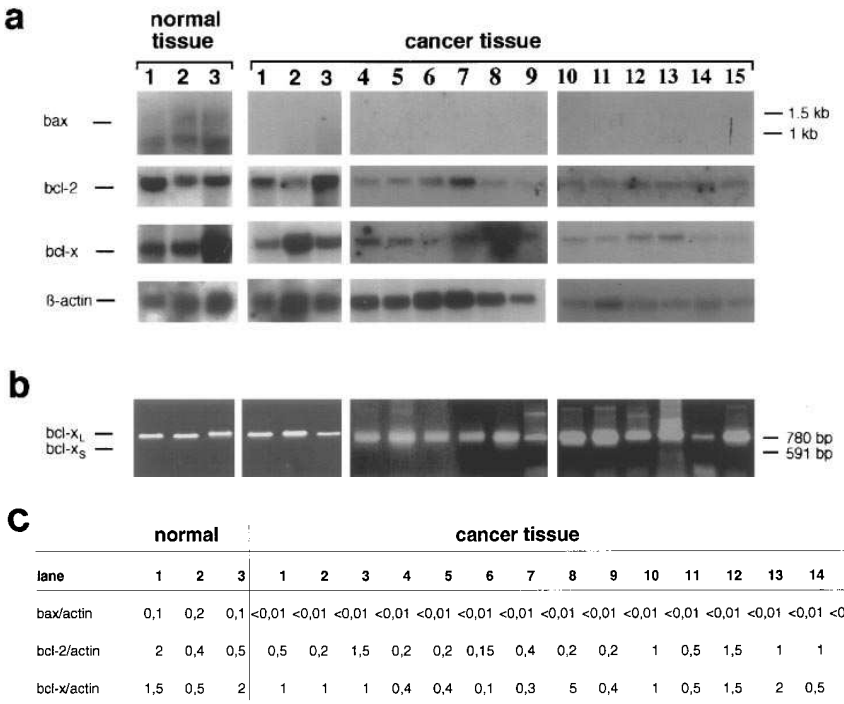
The bax-α full-length cDNA was derived from RT-PCR (see below). PCR fragments were sequenced and cloned into the expression plasmid pUHD 10-3 (34). This plasmid was cotransfected with the resistance plasmid pKEX (hygromycin resistance) (35) in a second step into the stable cell lines.

Modulation of bax-α expression was induced by incubating the cells in medium with (2 μg/ml) or without tetracycline. Additionally, using the same protocol, mock transfectants containing no insert in pUHD 10-3 were generated.

mRNA preparation and Northern blotting. RNA preparation was performed using the guanidium isothiocyanate (GITC)/CsCl method. Briefly, cells or tissue were lysed with GITC solution (4 M GITC, 20 mM sodium acetate, pH 5.2, 0.1 mM DTT, 0.5% sarcosyl [Sigma Chemicals, Grünwald, Germany]). The resulting GITC cell lysate was layered on top of a CsCl cushion (5.7 M, 100 mM EDTA). After ultracentrifugation at 150,000 *g* for 23 h at 18°C the RNA pellet was resuspended in Tris-EDTA (Tris 10 mM, EDTA 5 mM, pH 7.4) and precipitated by 3 M sodium acetate and ethanol at −80°C for 30 min. 10 μg of RNA was loaded onto a 1.2% agarose/1.1% formaldehyde gel and separated electrophoretically. Northern transfer of RNA to nylon membrane was performed by vacuum blotting (Appligene). After prehybridization (50% deionized formamide, 0.6 M NaCl, 0.04 M NaH_2PO_4, pH 7.4, 4 mM EDTA, 1% SDS, 100 μg/ml herring sperm DNA) at 42°C for 4–16 h, blots were hybridized for 20 h with a ^{32}P-random prime labeled probe.

RT-PCR analysis and human cDNA probes. Total RNA was extracted from cells and tissues as described above. Purification of poly(A)$^+$ RNA from 10 μg of total RNA was performed using the Dynabead mRNA purification kit (Dynal, Oslo, Norway). Detection

1. *Abbreviation used in this paper:* ER, estrogen receptor.



lane	1	2	3	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
bax/actin	0,1	0,2	0,1	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01	<0,01
bcl-2/actin	2	0,4	0,5	0,5	0,2	1,5	0,2	0,2	0,15	0,4	0,2	0,2	1	0,5	1,5	1	1	1,5
bcl-x/actin	1,5	0,5	2	1	1	1	0,4	0,4	0,1	0,3	5	0,4	1	0,5	1,5	2	0,5	0,5

Figure 1. mRNA expression of bcl-2, bcl-xL, bcl-xs, and bax in breast cancer tissue. (*a*) Shown is Northern blot hybridization of three different normal tissue samples (*lanes 1–3*) and 15 different cancer tissue samples (*lanes 1–15*). Normal tissue (*lanes 1–3*) was derived from nonmalignant areas (as defined by histology) of the same breast as the malignant tissue (*lanes 1–3*). Cancer-derived cell lines show low bax expression. In cancer tissue samples no bax signal can be detected. In contrast, strong expression is found in all normal cell lines and tissues. bcl-2 and bcl-x are expressed in all tissue samples, β-actin served as a control. Note the shorter exposition time of the right β-actin blot (*lanes 10–15*). (*b*) RT-PCR analysis of bcl-x. PCR primers were designed to detect the long (bcl-xL) and short (bcl-xs) splice variant simultaneously generating PCR bands of 780 and 591 bp, respectively. The same tissue samples (*lanes 1–15*) as in *a* were analyzed. bcl-xs mRNA could only be detected in R30C cells. (*c*) Northern data on normal (*lanes 1–3*) and malignant (*lanes 1–15*) primary tissue were quantified by using an Instant Imager (Canberra Packard). The ratio of bax/actin, bcl-2/actin, and bcl-x/actin was determined.

Table I. Expression of Members of the bcl-2 Family in Normal and Malignant Breast Tissue

Histology	bcl-2	bcl-xL	bcl-xs	bax-α
Ductal invasive	31/33	33/33	0/33	1/33
Lobular	2/2	2/2	0/2	0/2
Intraductal	4/4	4/4	0/4	0/4
Mucinous	1/1	1/1	0/1	0/1
Fibrocystic	4/4	4/4	0/4	4/4
Normal	6/6	6/6	0/6	6/6

Northern blot and RT-PCR data on bcl-2, bcl-xL, bcl-xs, and bax expression are summarized (see also Fig. 1). RT-PCR was used to be able to discriminate between the long (bcl-xL) and short (bcl-xs) splice variant of bcL-x (see also Fig. 1 *b*). bax expression was additionally determined by immunohistology (see also Fig. 2). 40 samples derived from breast cancer tissue, four cases of fibrocystic disease and tumor-free tissue (as defined by histology) of six different cancer samples were analyzed. Malignant tissue samples included different histological subtypes: 33 ductal invasive, 2 lobular invasive, 1 mucinous, and 4 intraductal (in situ carcinoma) carcinomas. Comparable amounts of bcl-2 and bcl-xL expression could be detected in normal and malignant breast epithelium, whereas bcl-xs expression could not be detected. No or only weak bcl-2 expression was detected in two cancer samples of the ductal invasive type, whereas bcl-xL expression could be detected in every tissue sample examined so far. Strong expression of bax is detectable in normal breast epithelium, whereas only partial, weak, or no expression could be found in 39 out of 40 different cancer tissue samples examined so far. Partial bax expression was observed in 23 tumor samples. In these cases 5–20% of the invasive component was bax positive. About the same ratio between positive and negative cells could be observed in noninvasive components (in situ carcinoma). 29 tumor samples contained bax-positive cells as an internal control (normal epithelium, malignant cells, or nonepithelial cells). Note that the seven cases that did not contain such internal control cells were also devoid of histologically normal tissue.

of mRNA by PCR was performed as described (36) using a Geneamp RNA PCR kit (Perkin Elmer-Cetus, Überlingen, Germany) and a thermocycler (Bachhofer, Reutlingen, Germany) according to the PCR protocol. In brief, 10 ng of poly(A)$^+$ RNA was used per experiment and 30 cycles were performed (conditions per cycle: 40 s at 94°C, 60 s at 60°C, 40 s at 72°C). bcl-x, bcl-2, and bax PCR products were cloned, sequenced, and subsequently used as cDNA probes for Northern blot hybridization as described before (32). Full-length bax cDNA was generated for expression experiments (see above).

To distinguish the bcl-x splice variants, poly(A)$^+$ RNA from the various cells and tissues was analyzed by RT-PCR. Up- and downstream primers were used to amplify bcl-xL and bcl-xs simultaneously (upstream: 5′-TTGGACAATGGACTGGTTGA-3′; downstream: 5′-GTAGAGTGGATGGTCAGTG-3′) resulting in bands of 780 and 591 bp, respectively. bcl-2 was amplified using the following primers: upstream: 5′-GGTGCCACCTGTGGTCCACCTG-3′, downstream: 5′-CTTCACTTGTGGCCCAGATAGG-3′ (resulting fragment length: 459 bp). bax-α was amplified using the following primers: upstream: 5′-CCGGAATTCCGGATGGACGGGTCCGGGGAGCAG-3′, downstream: 5′-TGCTCTAGAGCATCAGCCCATCTTCTTCCAG-3′ (resulting fragment length: 579 bp). β-actin was amplified using the following primers: upstream: 5′-GAGCTGCGTGTGGCTCCCG-AGG-3′ (resulting fragment length: 579 bp), downstream: 5′-CG-CAGGATGGCATGGGGGAGGGCATACCCC-3′ (resulting fragment length: 246 bp).

Protein preparation and immunoblotting. Cell lysates were obtained by incubating 10^7 cells in lysis buffer (20 mM Tris-acetate, pH 7, 10 mM sodium-glycerophosphate, 50 mM sodium-fluoride, 5 mM sodium-pyrophosphate, 1% Triton X-100, 0.1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 0.27 mM sucrose, 2 μg/ml leupeptin) for 30 min at 4°C. Thereafter lysates were centrifuged for 10 min (1,000 *g*). The supernatant containing the membrane and cytoplasmic proteins was precipitated using methanol/chloroform. 20 μg protein per lane was electrophoretically separated using 12% SDS-PAGE. Detection of bcl-2, bax, or tubulin protein by immunoblotting or immunohistology (see below) was performed using the following antibodies: anti-bax: polyclonal rabbit antiserum purchased from Santa Cruz Biotech-

nology, Inc. (Santa Cruz, CA); anti–bcl-2: monoclonal mouse antibody purchased from Sigma Chemicals; anti-tubulin: monoclonal rat antibody purchased from Serotec (Wiesbaden, Germany). After transfer to nitrocellulose and blocking (3% BSA 1× PBS, overnight 4°C), filters were incubated with antiserum (dilution 1:200 [anti-bax], 1:1,000 [anti–bcl-2 or anti–bcl-x] in 1× PBS, 1% BSA, 0.5% Tween 20). Thereafter filters were incubated with alkaline phosphatase–conjugated goat anti–rabbit or goat anti–mouse antibody (Dianova, Hamburg, Germany; dilution 1:7,500 in 1× PBS, 1% BSA, 0.5% Tween 20) for 40 min at room temperature. Bands were visualized using chromogenic substrates NBT/BCIP (Roth, Karlsruhe, Germany).

Immunohistology. From the quick-frozen tissues frozen sections of ~ 4–6 μm in thickness were air dried, fixed in acetone for 10 min at room temperature, and immunostained immediately or stored at −20°C. Antibodies against bax or bcl-2 are described above and were used for immunohistology. For antibody staining, an alkaline phosphatase/anti–alkaline phosphatase staining procedure was applied as described before (38). The sections were counterstained with hematoxylin.

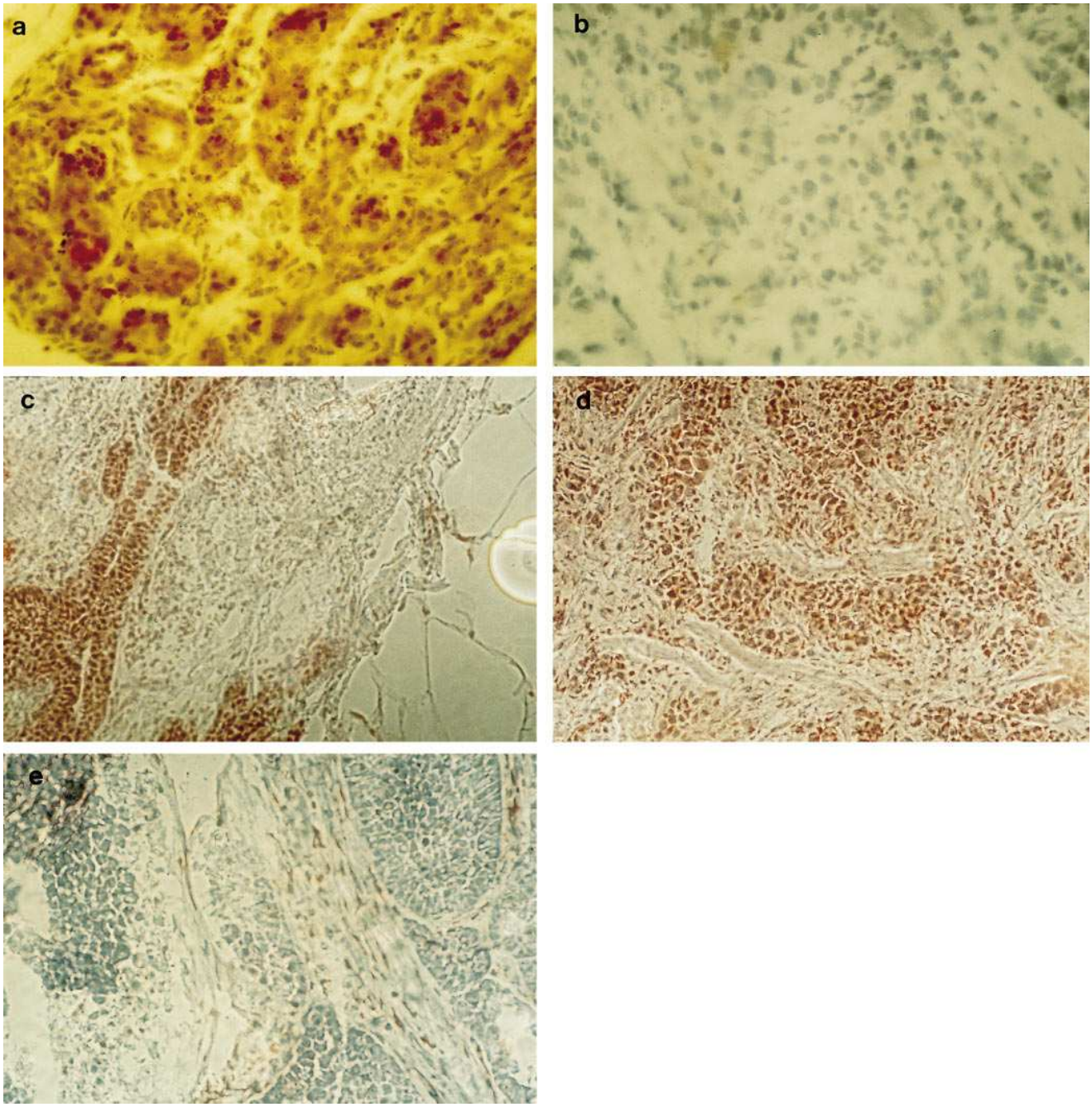


Figure 2. Immunohistological analysis of bax expression in normal and malignant breast epithelium. Shown is bax and bcl-2 staining of frozen tissue sections. Anti-bax or anti–bcl-2 staining is visualized via an indirect alkaline phosphatase method. A faint hematoxylin staining serves as counterstain. (*a*) Lobulo-alveolar structure normal breast epithelium. Strong bax staining is detectable in nearly every epithelial cell. (*b*) Malignant epithelium of a ductal invasive carcinoma. No bax expression is detectable in the majority of the cells. (*c*) Ductal epithelium of a patient with fibrocystic disease. Strong bax staining is detectable in every epithelial cell. (*d*) Malignant epithelium of a ductal invasive carcinoma. Strong bcl-2 staining is detectable. (*e*) Malignant epithelium of a ductal invasive carcinoma with an intraductal portion. No bax expression is detectable.

Table II. Comparison between mRNA Expression Levels of Members of the bcl-2 Family in Breast Cancer Tissue and Other Tumor/Patient Characteristics

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
bax/actin	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
bcl-2/actin	0.5	0.2	1.5	0.2	0.2	0.15	0.4	0.2	0.2	1	0.5	1.5	1	1	1.5
bcl-x/actin	1	1	1	0.4	0.4	0.1	0.3	5	0.4	1	0.5	1.5	2	0.5	0.5
Postmenopausal	+	−	+	+	−	+	−	+	−	−	−	−	−	+	+
ER	−	−	+	−	+	−	−	+	+	−	+	−	−	−	+
PR	+	+	−	+	−	−	−	−	+	−	−	−	−	+	+
p53	−	−	−	+	−	−	−	−	+	+	−	−	+	−	−

Quantitative mRNA analysis of bcl-2, bcl-x, and bax expression of 15 patients is also shown in Fig. 1 *c*. *PR*, progesterone receptor. p53 status was determined by immunohistology as described in Methods.

Expression of p53 was evaluated according to previously described methods (39) using a monoclonal anti-p53 antibody (PAb1801; Cambridge Research Biochemicals, Wilmington, DE).

Xenotransplantation of cells into SCID mice. R30C and MCF-7 cells (10^6) were injected subcutaneously into C.B.-17 scid/scid mice. The mice were obtained from our own breeding colony and were kept in isolators under stringent conditions in the central animal laboratory of the Max Delbrück Center for Molecular Medicine. Microbiological controls were performed regularly by addition of sterile sentinel animals to the colony. Tumors were measured in two dimensions, length (a) and width (b), by means of calipers in millimeters. Tumor volume (V) was calculated according to $V = ab^2/2$, where a is the longer of the two measurements.

Results

bcl-x and bcl-2 are expressed in normal and malignant breast epithelium. Expression of bcl-x and bcl-2 was studied in 40 different breast cancer tissue samples and 10 nonmalignant breast tissue samples by Northern blot analysis. Nonmalignant tissue samples included four cases of fibrocystic disease and tumor-free tissue of six different cancer samples. To discriminate between the two bcl-x splice variants we additionally performed RT-PCR analysis. No major difference was observed with regard to bcl-2 or bcl-xL expression between normal and malignant breast tissue (Fig. 1 and Table I). No or only weak bcl-2 expression could be detected in two cancer samples of the ductal invasive type (Table I). bcl-xs expression could not be detected, neither in normal nor in malignant breast epithelia.

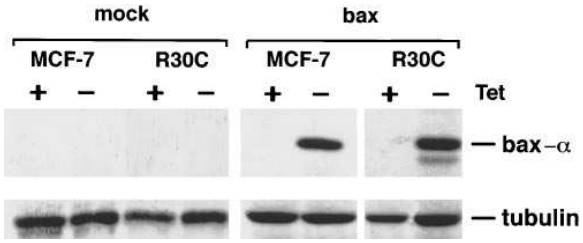


Figure 3. Inducible expression of bax-α in breast cancer cell lines. Shown is Western blot analysis of bax-α protein expression in MCF-7 and R30C breast cancer cells. Mock transfectants and stable bax clones, which are under transcriptional control of a tetracycline-dependent repressor, were generated as described in Methods. Cells were cultured in the presence or absence of tetracycline. Protein was extracted 24 h after tetracycline withdrawal. Equal amounts of protein (20 μg) were loaded on each lane. Strong induction of bax-α protein can be detected after tetracycline withdrawal, whereas in the presence of tetracycline bax expression is completely repressed. Tubulin served as a control.

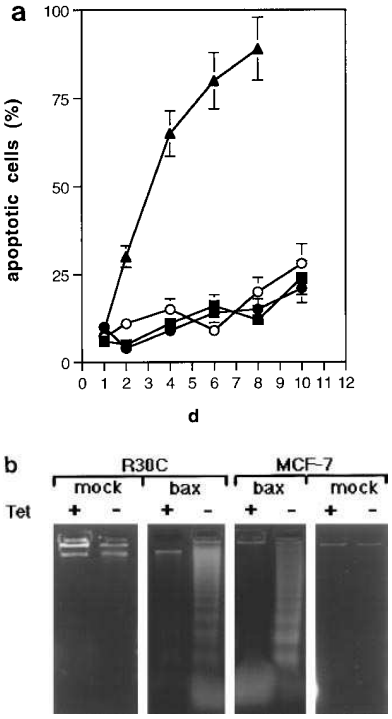


Figure 4. Induction of apoptosis in bax-expressing R30C and MCF-7 clones after serum starvation. Untransfected (R30C), mock-transfected (R30C mock), or bax-transfected cells (R30C bax, MCF-7 bax) were cultured in the presence or absence of tetracycline (+/− Tet) for prolonged time periods after serum starvation. (*a*) Apoptosis was assayed morphologically by staining the nuclei with acridine orange at days 1, 2, 4, 6, 8, and 10. The number of fragmented nuclei (indicating apoptosis) from 100 cells was determined and expressed as a percentage of apoptotic cells. The mean and standard deviation of four independent experiments is shown. Induction of bax by tetracycline withdrawal strongly increases sensitivity towards apoptosis in R30C breast cancer cells. Apoptosis sensitivity was comparable with that found in cultured cells which were derived from nonmalignant breast epithelium (32). *Filled circles*, R30C; *filled squares*, R30C mock; *open circles*, R30C bax + Tet; *filled triangles*, R30C bax − Tet. (*b*) Detection of apoptosis by DNA fragmentation in R30C and MCF-7 cells. Inducible bax transfectants of R30C or MCF-7 were cultured in the presence (+) or absence (−) of 2 μg/ml tetracycline. 24 h after tetracycline withdrawal serum was depleted. Agarose gel electrophoresis of DNA extracted from R30C and MCF-7 cells was performed 3 d after serum starvation. Mock transfected R30C and MCF-7 cells served as a control.

Additionally, we quantified Northern data of 15 patients using an Instant Imager. The ratio of bax/actin, bcl-2/actin, and bcl-x/actin was determined (Fig. 1 *c*). Quantification revealed differences in bcl-2 and bcl-x mRNA expression levels between different tumor samples. However, different bcl-2 and bcl-x expression levels could also be observed in normal breast tissue in a similar range as in tumor tissue. There are some tumors which showed bcl-2 and bcl-x expression levels beneath the range of normal tissue. One tumor showed elevated bcl-x expression compared with normal tissue. However, the majority of tumor tissue samples analyzed so far revealed similar amounts of bcl-2 or bcl-xL compared with normal mammary tissue.

bax-α is strongly expressed in normal breast epithelium but is downregulated in malignant epithelia. Expression of bax mRNA and protein was studied in normal and malignant breast epithelium (see above) by Northern blot hybridization and immunohistology. Nonmalignant tissue samples included four cases of fibrocystic disease and tumor-free tissue of six different cancer samples. bax-α was expressed in high amounts in normal breast epithelium, whereas only partial, weak, or no expression could be detected in the vast majority of cancer tissue samples examined so far (Figs. 1 and 2, all tissue data are summarized in Table I). Thus, data on downregulation of bax expression on mRNA level in breast cancer tissue could be confirmed by immunohistological analysis.

In addition to the 1-kb splice variant which encodes the functional death-promoting bax-α protein, a longer 1.5-kb mRNA which is consistent with bax-β could be detected in normal tissue. The function of this variant which is probably not translated into protein is unknown.

Only 1 out of 40 cancer tissue samples examined so far revealed a bax expression level comparable with that found in normal epithelium (Table I). Interestingly, downregulation of bax-α was found in different histological subtypes, including ductal invasive, lobular, mucinous, and even intraductal (carcinoma in situ) carcinomas (Table I and Fig. 2).

It has been reported that p53 might be involved in the transcriptional regulation of bax expression (15). Furthermore, it has been published that estrogens may modulate bcl-2 expression in breast tissue (40). Therefore, we provided data on the hormonal status (pre- versus postmenopausal, ER, progesterone receptor) and the p53 status and compared these data with bcl-2, bcl-x, and bax mRNA expression levels in different breast cancer tissues (Table II). Differences in bcl-2 and bcl-x mRNA expression levels as well as the strong downregulation of bax in breast cancer tissue did not correlate with the hormonal status or the p53 status. The lack of a correlation between p53 status and bax expression was also reported by Krajewski et al. (41).

Induction of bax-α expression in breast cancer cell lines can restore sensitivity of serum starvation or APO-I/Fas–mediated apoptosis. Recently, we have shown that low bax-α expression in breast cancer cell lines correlates with resistance towards apoptosis (32). In contrast to malignant cell lines, which express low levels of bax-α, nonmalignant epithelial cell lines displaying high amounts of bax-α were highly sensitive to induction of programmed cell death by both serum starvation and APO-I/Fas (CD95) triggering (32). To test whether low bax-α expression in breast cancer cell lines is due to apoptosis resistance we transfected bax cDNA under the control of a tetracycline-regulated expression system into these cells. As shown in Fig. 3, withdrawal of tetracycline led to strong induction of bax expression in R30C and MCF-7 cells. In the presence of tetracycline bax expression was completely repressed. As depicted in Fig. 4, *a* and *b*, induction of bax-α expression strongly increases sensitivity towards apoptosis induced by serum starvation. bax-expressing cells started to die within 2 d, whereas clones which were devoid of bax expression showed prolonged resistance to apoptosis at least until day 10 after serum starva-

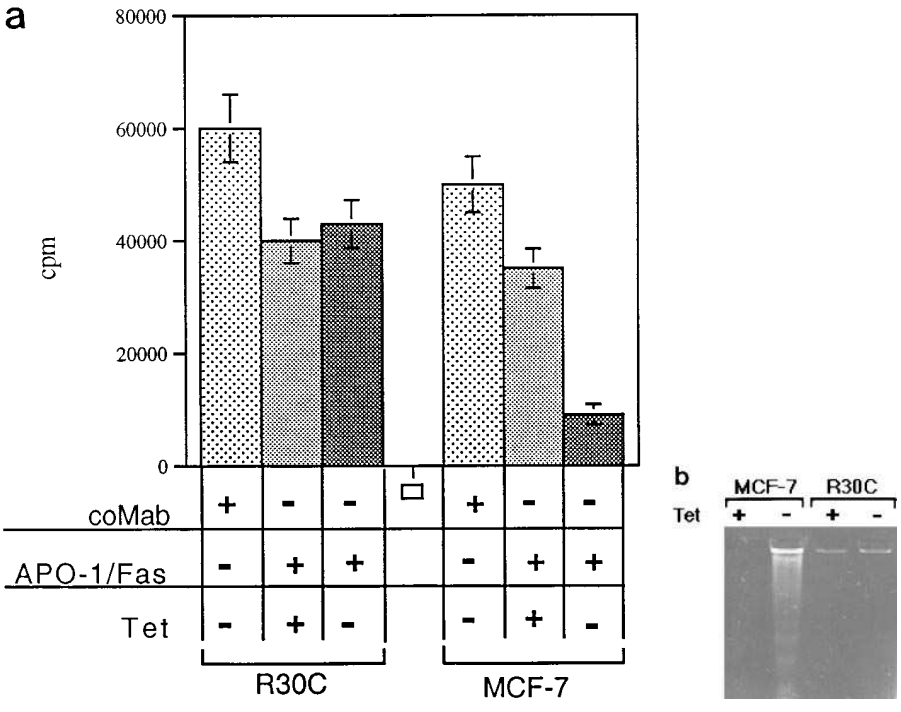


Figure 5. Induction of apoptosis in bax expressing R30C and MCF-7 clones after APO-1/Fas triggering. (*a*) Antiproliferative effect of anti–APO-1 on R30C and MCF-7 breast cancer cells. Inducible bax transfectants of R30C or MCF-7 were cultured in the presence (+) or absence (−) of 2 μg/ml tetracycline. 24 h after tetracycline withdrawal 1 μg/ml anti–APO-1 or FII23c was added to the culture medium. [^{3}H]Thymidine incorporation assay was performed 48 h after APO-1/Fas triggering as described in Methods. The mean and standard deviation of four independent experiments is indicated. (*b*) Detection of apoptosis by DNA fragmentation in R30C and MCF-7 cells. Inducible bax transfectants of R30C or MCF-7 were cultured in the presence (+) or absence (−) of 2 μg/ml tetracycline. 24 h after tetracycline withdrawal 1 μg/ml anti–APO-1 was added to the culture medium. Agarose gel electrophoresis of DNA extracted from R30C and MCF-7 cells was performed 2 d after APO-1/FAS triggering. DNA fragmentation can only be observed in bax-expressing MCF-7 cells after tetracycline withdrawal.

tion. Apoptosis sensitivity in bax-expressing cancer cell lines was comparable with that found in HMEC and HBL-100 cells (32), which were derived from normal (nonmalignant) human breast epithelium.

Furthermore, we could show that induction of bax-α in the breast cancer cell line MCF-7 strongly increases sensitivity towards APO-1/Fas–mediated apoptosis (Fig. 5, *a* and *b*). A weak apoptotic effect of APO-1/Fas triggering (30% growth inhibition) could also be observed in cells which were devoid of detectable bax expression levels. In contrast, induction of bax expression did not affect resistance to APO-1/Fas–mediated apoptosis in R30C cells, indicating that bax expression is important but not sufficient to confer APO-1/Fas sensitivity.

Thus, we showed that induction of bax-α can restore sensitivity towards different apoptotic stimuli in breast cancer cell lines.

Induction of bax-α expression reduces tumor growth in SCID mice. To test the effects of bax expression on tumor growth in vivo, we transplanted bax-expressing clones of the breast cancer cell lines R30C and MCF-7 into SCID mice. As shown in Fig. 6, bax-expressing breast cancer cells are still tumorigenic. However, tumor size and velocity of tumor growth are significantly reduced compared with mock transfected or untransfected clones.

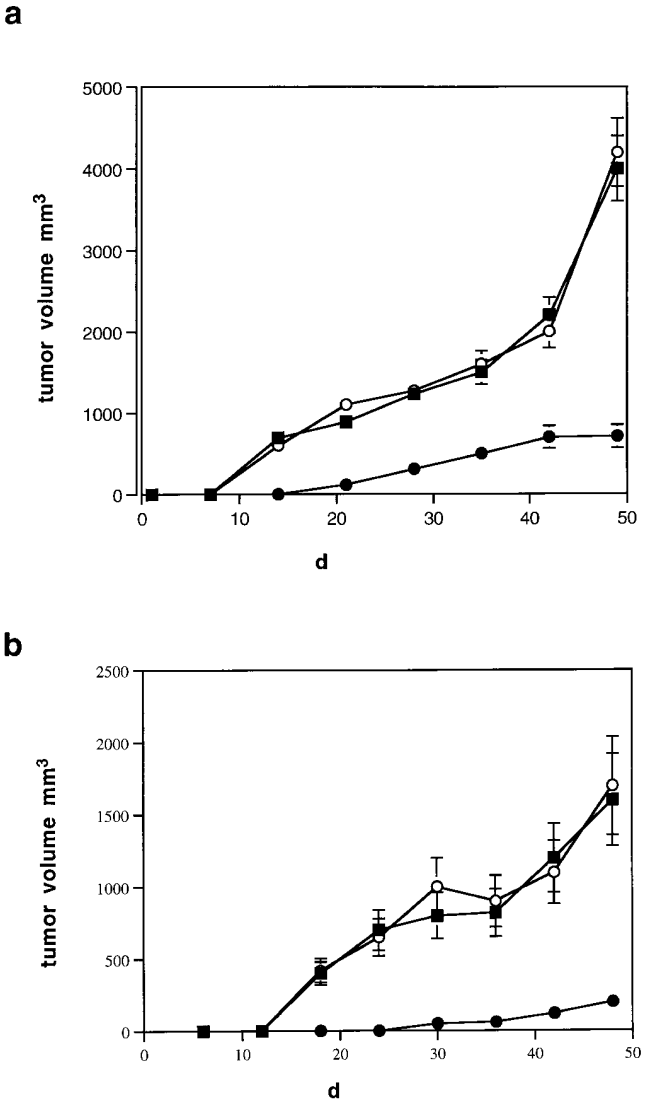


Figure 6. Tumor growth of bax-expressing R30C and MCF-7 clones in SCID mice. Shown is tumor growth of five different bax-expressing and five different mock clones of R30C and MCF-7 breast cancer cells. Untransfected clones were included. Each cell clone was transplanted on six different animals. Animals were challenged with equal cell numbers (10^6) at day 0. Tumor size was determined at different time points as described in Methods. Mean and standard deviation of 30 (R30C bax, R30C mock, MCF-7 bax, MCF-7 mock) or 6 (R30C, MCF-7) independently performed experiments are shown. Symbols in *a*: *filled circles*, R30C bax; *filled squares*, R30C mock; *open circles*, R30C. Symbols in *b*: *filled circles*, MCF-7 bax; *filled squares*, MCF-7 mock; *open circles*, MCF-7.

Discussion

Apoptosis is a physiological process that contributes to the regulation of development, differentiation, and growth in various tissues. Therefore, dysregulation of apoptosis might be involved in pathogenesis of neoplasias. This is particularly well documented for malignant lymphomas where bcl-2 overexpression was shown to be involved in malignant transformation (42).

In carcinomas, the role of bcl-2 in tumorigenesis is still unclear. We and others (29, 32) have observed bcl-2 expression in breast cancer without a marked difference to normal epithelium. Some tumors failed to express bcl-2, which surprisingly correlated with markers of poor prognosis (29). Similar results were obtained for non–small cell lung carcinoma (43). bcl-xL expression appeared to be elevated in some tumors and reduced in others. However, in accordance with the data concerning bcl-2, the majority of tumor tissue samples analyzed so far revealed similar amounts of bcl-xL compared with normal mammary tissue. bcl-xs expression could not be detected, neither in normal nor in malignant breast epithelia.

In contrast, analysis of bax expression revealed a marked difference between normal and cancer epithelia: strong expression of bax-α was observed in normal epithelial cells and breast tissue, whereas only weak or partial expression was found in their malignant counterparts. Low levels of bax in conjunction with normal bcl-2 and bcl-x levels might disrupt the cellular homeostasis leading to an accumulation of cells, which might thus become susceptible for secondary mutagenic events resulting in malignant transformation. Since differences in bcl-x and bcl-2 expression between normal and some malignant tissue samples could be found, we cannot exclude that in some cases deregulation of bcl-2 or bcl-x expression might also modulate apoptosis in breast cancer cells. However, compared with the striking change in bax expression, these differences are presumably of minor significance.

As low bax expression is still found in some tumors and cell lines, weak bax expression is probably not due to gross genetic alterations of the bax locus itself. It is conceivable that other genes involved in malignant transformation might cause downregulation of bax. p53 itself or p53-regulated genes could be candidates, because p53 has also been shown to be a regulator of apoptosis and a direct transcriptional activator of the human bax gene (13–15, 44). Thus, constitutive expression of bcl-2 and bcl-xL and low expression of bax might at least in part be due to a loss of function of p53. However, since we found no correlation between bax downregulation and p53 status, we assume that besides p53 further genes are involved in the downregulation of bax expression in malignant breast epithelium.

The CD95 antigen and its role in apoptosis have been studied mainly in normal and malignant lymphoid cells. However, CD95 can also be detected in many epithelial tissues (24). In these experiments we provide evidence that, as in lymphoid cells, expression of CD95 does not necessarily correlate with sensitivity towards apoptosis (45). In B-CLL cells an inverse correlation between bcl-2 expression and sensitivity to CD95-mediated apoptosis was found (45). In contrast, sensitivity to CD95-mediated apoptosis does not correlate with bcl-2 expression in prostatic adenocarcinoma or colon carcinoma cell lines (46) and overexpression of bcl-2 is able to confer only partial protection against CD95-mediated apoptosis (47). Thus, additional genes have to be involved in regulation of CD95-triggered apoptosis. Recently, it has been shown that coexpression of bcl-2 and bag-1, another apoptosis-regulating gene, which does not belong to the bcl-2 gene family, completely protects from CD95-mediated apoptosis (48).

Recently, we have shown that low bax-α expression in breast cancer cell lines correlates with resistance towards apoptosis (32). In contrast to malignant cell lines, which express low levels of bax-α, nonmalignant epithelial cell lines displaying high amounts of bax-α were highly sensitive to induction of programmed cell death by both serum starvation and APO-I/Fas (CD95) triggering. To test whether low bax-α expression in breast cancer cell lines is due to apoptosis resistance, we transfected bax cDNA under the control of a tetracycline-regulated expression system into these cells. We were able to show that induction of bax expression in breast cancer cell lines restores sensitivity towards both serum starvation or CD95-induced apoptosis. Thus, we were able to demonstrate for the first time that bax can confer sensitivity to different apoptotic stimuli, e.g., serum starvation or CD95 triggering. Therefore, it appears that the bcl-2 family constitutes a major death checkpoint integrating different apoptotic pathways. The decreased sensitivity of breast carcinoma cell lines towards CD95-mediated apoptosis furthermore indicates a potential role of an intact CD95 apoptosis signaling pathway in the homeostasis of normal breast epithelium.

Consistent with our observations, another recent paper demonstrated reduced bax immunostaining in breast cancers and found correlations with poor responses to chemotherapy and patient survival (41). The authors arbitrarily defined bax-negative cases as those in which the vast majority of the tumor cells were unstained ($< 10\%$), with bax-positive cases representing all others ($> 10\%$), and found that complete loss ($< 10\%$) of bax expression is correlated with poor response rates to chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. Since antineoplastic drugs can kill at least in part tumor cells via apoptosis, our functional data, showing that induction of the bax gene can restore sensitivity to different apoptotic stimuli in cultured breast cancer cells, might serve as an explanation for this correlation. In addition, the authors report that they could not detect reduced bax expression in in situ carcinomas, which is in contrast to our data. This contradiction might be due to different immunohistological staining techniques.

To test whether induction of bax-α affects tumor growth in vivo, we transplanted a bax-expressing breast cancer cell line into SCID mice. Although tumor growth could not be completely prevented, we were able to show that bax expression led to a significant reduction of tumor growth. This finding provides further evidence that dysregulation of apoptosis, at least in part due to downregulation of bax-α expression, contributes to the pathogenesis of breast cancer.

In summary, we could show the following: (*a*) bax-α expression is downregulated in malignant breast epithelium in comparison to normal epithelium; (*b*) induction of bax-α restores sensitivity towards serum starvation or APO-1/Fas–mediated apoptosis in breast cancer cell lines; and (*c*) induction of bax-α reduces tumor growth in SCID mice. Therefore, we propose that the bcl-2 family and APO-1/Fas are involved in the regulation of tissue homeostasis not only of the lymphoid system but also of epithelial cells. An imbalance within the bcl-2 family members leads to dysregulation of apoptosis and therefore might contribute to the pathogenesis of breast cancer.

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