Analysis of Bax and Bcl-2 Expression in p53-immunopositive Breast Cancers[1]

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ABSTRACT

Bax and Bcl-2 are proteins that regulate programmed cell death and apoptosis. The expression of these proteins can be regulated, at least in part, by the tumor suppressor p53, but the effects of p53 are highly tissue specific. In an effort to better understand the relation between p53 and the *in vivo* control of the expression of Bax and Bcl-2 in adenocarcinomas of the breast, we evaluated by immunohistochemistry the expression of Bcl-2 and Bax in 149 invasive ductal carcinomas, 135 of which were chosen because of their p53 immunopositivity. The percentages of Bcl-2-immunopositive tumor cells were significantly lower in the p53-positive (median 20%) subsets as compared to the p53-negative (median 85%) subsets ($P = 0.004$). Comparisons of the percentages of p53-immunopositive tumor cells with the percentages of Bcl-2- and Bax-positive cells (as continuous variables) revealed a significant inverse correlation between Bcl-2 and p53 ($r = -0.41$, $P < 0.001$) but not between Bax and p53. In the p53-positive subset, the percentages of Bax- and Bcl-2-immunopositive tumor cells were correlated positively ($r = 0.27$, $P = 0.002$), suggesting that the expression of these genes may be co-regulated to some extent in these breast cancers. Higher percentages of Bcl-2-positive tumor cells were also associated with estrogen receptor positivity ($P = 0.03$), low histological tumor grade ($P = 0.03$), and low T stage ($P = 0.02$), whereas Bax immunostaining was associated only with c-*erb*B-2 immunopositivity ($P = 0.02$). Although the number of cases was small and treatment was non-uniform, preliminary correlations with clinical outcome data suggest that the prognostic significance of Bcl-2 may be enhanced by inclusion of Bax data in patients with p53-immunopositive adenocarcinoma of the breast, at least for patients with node-negative disease. Taken together, these data suggest that, despite the ability of p53 to bind directly to the *Bax* gene promoter, the regulation of Bax in human breast cancers does not necessarily correlate with p53 status, implying that regulation of this pro-apoptotic gene in these tumors is complex and probably influenced by several factors.

INTRODUCTION

The development and growth of tumors are controlled by a combination of cellular replication and cell death (1). The most common mechanism of cell death is programmed cell death, a precisely regulated physiological process that culminates in a set of characteristic morphological and structural alterations referred to as apoptosis (2). Many apoptotic stimuli induce cell death through a pathway that is regulated by members of the Bcl-2 protein family. These proteins function either as promoters or inhibitors of cell death and have been shown to physically interact with each other by the formation of homo- and heterodimers (reviewed in Refs. 3–5). Bcl-2, Bcl-X_L, and Mcl-1, for example, are suppressors of apoptosis and can heterodimerize with Bax, a pro-apoptotic member of this family of homologous proteins (6–9).

WT[4] p53 protein is a DNA-binding transcriptional regulator that contributes to tumor suppression by inducing both cell cycle arrest and apoptosis (reviewed in Refs. 10 and 11). Loss of p53 function through gene deletion, mutations, or a combination of the two occurs frequently in advanced breast cancers and has been correlated with poor clinical outcome in some groups of patients (12, 13). Recent data indicate that WT p53 can transcriptionally repress Bcl-2 expression while inducing expression of Bax (14, 15). Using a temperature-sensitive version of p53, for example, conditional restoration of p53 activity in a p53-deficient leukemia cell line resulted in decreased Bcl-2, increased Bax, and stimulated apoptosis (14, 15). Similar results were obtained in p53 knock-out (transgenic) mice; absence of p53 was associated with increased Bcl-2 and decreased Bax in some tissues (14). The promoter of the human *Bax* gene has been shown to contain several consensus sequences for WT p53 binding and is strongly transactivated by WT p53 in reporter gene assays (16). Thus, the effects of WT p53 on apoptosis may be mediated in part through its effects on the expression of Bcl-2 and Bax.

In p53 knock-out mice, however, the absence of p53 alters expression of Bcl-2 and Bax only in some tissues but not others,

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[4] The abbreviations used are: WT, wild type; MGH, Massachusetts General Hospital; ER, estrogen receptor; DFS, disease-free survival; OS, overall survival; LR, likelihood ratio; RR, relative risk.

consistent with the idea that p53 is probably just one of many transacting factors that contribute to the overall regulation of the expression of these genes *in vivo* (14). Moreover, transfection of a mutant form of p53 into the WT p53-containing breast cancer cell line MCF7 cells has been reported to repress Bcl-2 expression, raising the possibility that p53 may regulate *Bcl-2* gene expression differently in breast cancers than in some other types of cells (17). In this regard, several studies have documented an inverse association between mutant p53 protein (as inferred from immunostaining data) and Bcl-2 immunopositivity (18–22). Although less is known about the relation between p53 and regulation of Bax expression in breast cancers, in a recent report no correlation was found when using $<10\%$ *versus* $\geq 10\%$ immunopositive cells as arbitrary cutoffs for dichotomizing the data (22).

The purpose of this report was to more closely examine the relations between p53, Bcl-2, and Bax immunostaining in human breast cancers. The approximate percentages of tumor cells with cytosolic Bax or Bcl-2 immunostaining were compared with the percentages of tumor cells with nuclear p53 immunostaining as continuous variables rather than using arbitrary percentage cutoffs. The tumors studied here were, therefore, purposely selected to provide a wide range of percentages of p53-immunopositive tumor cells (0.5–95%), thus providing an opportunity to compare the percentages of p53-positive tumor cells with the percentages of Bax- and Bcl-2-positive cells as continuous variables, rather than using arbitrary percentage cutoffs as in previous studies. A clinical spectrum of infiltrating ductal type breast cancers was studied, including both node-negative and -positive disease.

As an additional goal of this study, immunoblotting was also performed to explore which isoforms of Bax and Bcl-2 are produced in human breast cancers. Both the *BAX* and *BCL-2* genes can produce different proteins through alternative mRNA splicing mechanisms, including the α, β, γ, and δ isoforms of Bax and the α and β isoforms of Bcl-2 (6, 23, 24). The functions of some of these variant proteins may be different from the most abundant forms of Bax and Bcl-2 found in tissues, p21-Bax-α and p26-Bcl-2-α (8, 25, 26), thus making an assessment of which isoforms are usually present in breast cancers important for interpretation of the biological significance of immunostaining results.

MATERIALS AND METHODS

Patient Specimens. Invasive ductal carcinomas of the breast were derived from the MGH tumor registry and originated from patients who presented between 1977 and 1982. From ~1600 patients, 496 were identified who had continued care and long-term follow-up at MGH. Clinical and pathobiological information obtained from the patients' medical records or clinicians included: menopausal status, age at diagnosis, location of primary tumor, time to local recurrence, time to metastases, metastatic sites, therapeutic interventions (surgical, chemotherapeutic, radiotherapeutic, and hormonal), overall survival time, cause of death, lymph node metastases, tumor size, nuclear grade, and biochemical estrogen receptor content. Criteria for exclusion included distant metastases at the time of diagnosis, only *in situ* disease, or a history of other malignancies. Immunohistochemical analysis of these MGH tumors revealed 135 specimens containing tumor cells with nuclear p53 immunopositivity. Mean and median follow-up for the 139 patients used in the survival analysis was 7.3 and 7.2 years, respectively. Fourteen histologically similar archival cases that were immunonegative for p53 were used as a p53-negative subgroup for comparative analysis with Bcl-2 and Bax but were not included for clinical outcome analysis. These 14 p53-immunonegative tumors were derived from the same cohort of patients as the p53-positive samples and were processed in the same manner.

Tumors were staged according the criteria of the American Joint Committee on Cancer (27). Lymph node-negative patients ($n = 53$) underwent either a modified radical mastectomy or a lumpectomy followed by radiation therapy. Lymph node-positive patients ($n = 67$) underwent either a modified radical mastectomy or a lumpectomy and radiation therapy followed by chemotherapy and/or hormone therapy. There were 15 lymph node-unknown patients who were treated with mastectomy or lumpectomy, with or without adjuvant radiotherapy, hormone therapy, or chemotherapy.

Immunohistochemical Analysis. The preparation and characterization of antisera specific for amino acids 43–61 of the human Bax protein and 41–54 of the human Bcl-2 protein have been described previously (22, 28). Formalin-fixed, paraffin-embedded, 4-μm tissue sections were immunostained for Bax or Bcl-2 as described in previous publications (22, 28–31) using biotinylated goat antirabbit IgG followed by an avidin-biotin complex reagent (Vector Labs, Inc.) and horseradish peroxidase-conjugated avidin. Colorimetric detection of bound antibody was achieved with diaminobenzidine, followed by counterstaining with hematoxylin. For each of these, internal positive control cells consisted of either carcinoma, lymphocytes, or benign breast epithelium. Each case which was used had some control cell staining for either Bcl-2 or Bax. The Bax antiserum was used at a 1:1500 (v/v) dilution. The approximate percentage of Bax- or Bcl-2-immunopositive cells was determined for the invasive component of the tumors by one of us (A. T.) scoring the entire slide. Four cases were excluded from Bax analysis (one because of the tissue detached from the slide during microwaving and three because of high background staining).

p53 staining was performed according to published methods (12). Briefly, 4-μm sections were deparaffinized, rehydrated through graded alcohols, rinsed in PBS, and stained overnight at 4°C with a 1:4000 dilution of monoclonal anti-p53 antibody PAb 1801 (Cambridge Research Biochemicals, Inc., Wilmington, DE). Visualization was performed using biotinylated horse-antimouse (Vector Labs, Inc.), streptavidin-HRP (Zymed Laboratories, Inc., San Francisco, CA), followed by diaminobenzidine (Sigma Chemical Co., St. Louis, MO). Sections were counterstained with 1% methyl green, followed by dehydration in graded alcohols; then sections were cleared in xylene and coverslipped with mounting media. Positive and negative cell line controls were included with each assay, and an assay substituting PBS without primary antibody or a mouse tumor antibody (MOPC-21; Bionetics Laboratory, Kensington, MD; 40 μg/ml) were also done on each case as reported previously (12). All of the specimens included in this study (as p53

immunopositive) contained at least three to five invasive tumor cells with unequivocal nuclear p53 immunopositivity visible at ×100. In cases where the percentage of p53-positive tumor cells was <1%, the data were truncated to 0.5% for database calculations. All of the tumors studied here had also been evaluated previously for ER status by biochemical assays and for c-*erb*B-2 by immunohistochemical methods, as described in detail previously (32). Immunopositivity was evaluated by microscopic examination of the entire slide mounted tissue section by A. T., and the percentage of immunopositive cells within the invasive component of the tumor was estimated. Staining that was not visible at ×100 was not considered positive.

Immunoblot Assays. Frozen breast cancers or normal breast tissue samples (stored at −70°C) were placed into liquid nitrogen and ground to a fine powder using a pre-cooled mortar and pestle. This material was then resuspended in 1 to 2 volumes of RIPA buffer containing various protease inhibitors as described (28–31) and thawed on ice. Samples were sonicated on ice using 1–3-s pulses and a 1.5-mm (diameter) tip, until nonviscous and clarified. After centrifugation at 16,000 × *g* for 20 min, the supernatant was transferred to a fresh tube, and the protein content of an aliquot of the samples was determined by the bicinchoninic acid method (33). Approximately 100 μg of total protein from each sample were subjected to SDS-PAGE/immunoblot analysis as described in detail previously (31), using 12% gels and 0.05–0.1% (v/v) anti-Bax antiserum, followed by biotinlyated goat antirabbit IgG, avidin-biotin-complex reagent containing horseradish peroxidase (Vector Labs, Inc.), and the substrate Vector SG.

Statistical Analysis. Parametric *t* tests and nonparametric Mann-Whitney tests were used to compare the distributions of Bcl-2 and Bax among different components of the tumor, *i.e.*, invasive, *in situ*, and nonneoplastic epithelium. For comparisons of Bcl-2 and Bax with other tumor characteristics, ANOVA was used to determine statistical significance. Contingency table analyses based on χ^2 statistics were used to determine the statistical significance of associations between categorical variables. For determination of factors related to disease-free and overall survival, a Cox proportional hazards model with Bcl-2 and Bax entered as continuous variables was used. Statistical calculations and tests were performed using either Statview 4 or Data Desk software and a Macintosh computer.

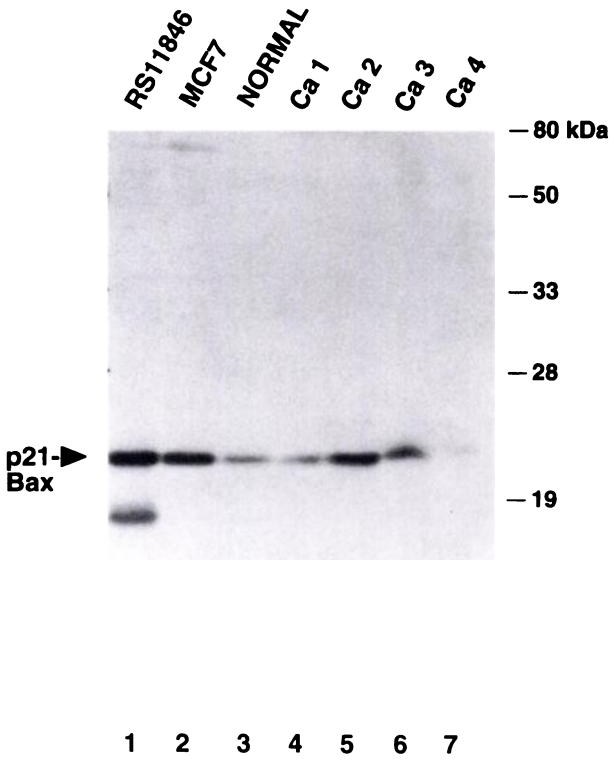

Fig. 1 Immunoblot analysis using Bax antiserum. Detergent lysates were prepared from RS11846 lymphoma cells, the MCF7 breast cancer cell line, normal mammary tissue, and four randomly chosen cases of breast cancer, normalized for total protein content (100 μg/lane) and subjected to SDS-PAGE (12% gel) before transfer to nitrocellulose. Filters were incubated with 0.1% (v/v) anti-Bax antiserum, and antibody-antigen complexes were detected by a colorimetric method. The blots were subsequently immunostained with anti-F_1-β-ATPase, verifying loading of approximately equivalent amounts of intact proteins for each sample (data not shown). The position of the p21-Bax protein is indicated. RS11846 cells also contained p18-Bax-δ. The percentages of Bax-immunostained invasive tumor cells in CA-1, -2, -3, and -4 were 60, 90, 85, and 5%, respectively.

RESULTS

Analysis of Bax and Bcl-2 Proteins in Breast Cancers by Immunoblotting. SDS-PAGE/immunoblot analysis was performed using proteins obtained from normal breast tissue (n = 2) and four randomly chosen cases of breast cancer. These immunoblot data demonstrated the presence of the M_r 21,000 Bax protein in all samples examined (Fig. 1 and data not shown). No cross-reactivity was seen with the Bcl-2, Bcl-X, and Mcl-1 proteins, which migrate at M_r 26,000, M_r 28,000–30,000, and M_r 38,000–41,000 in this gel system (28–31). The M_r 25,000 β-, M_r 5,000 γ-, and M_r 18,000 δ isoforms of Bax were not detected in normal or malignant breast tissues. A M_r 18,000 protein, which may represent the δ isoform of Bax, however, was seen in RS11846, a lymphoma cell line that was included in the blot shown in Fig. 1 as a control. Immunoblot analysis of the same samples using anti-Bcl-2 antiserum revealed the presence of only the M_r 26,000 α form of Bcl-2. No M_r 22,000 Bcl-2-β protein was detected (data not shown). Because the specificity of the anti-Bcl-2 antiserum used for these studies has been confirmed by several previous publications (28–31, 34), only the data for Bax are presented here. These data thus indicate that p21-Bax-α and p26-Bcl-2-α are the only isoforms of these proteins expressed in human breast cancers and further confirm the monospecificity of the Bax antibody developed in our laboratory. Although the samples were all normalized for total protein content, these data should not be considered quantitative, given the cellular heterogeneity of the samples, which contain not only malignant tumor cells but also various proportions of residual carcinoma *in situ*, normal mammary epithelium, fibroblasts, infiltrating lymphocytes, and endothelial cells.

Immunolocalization of Bax and Bcl-2 in Breast Carcinomas. An immunohistochemical approach was used to localize the Bax and Bcl-2 proteins in 145 cases of adenocarcinoma of the breast, all of which were infiltrating ductal type. Fig. 2

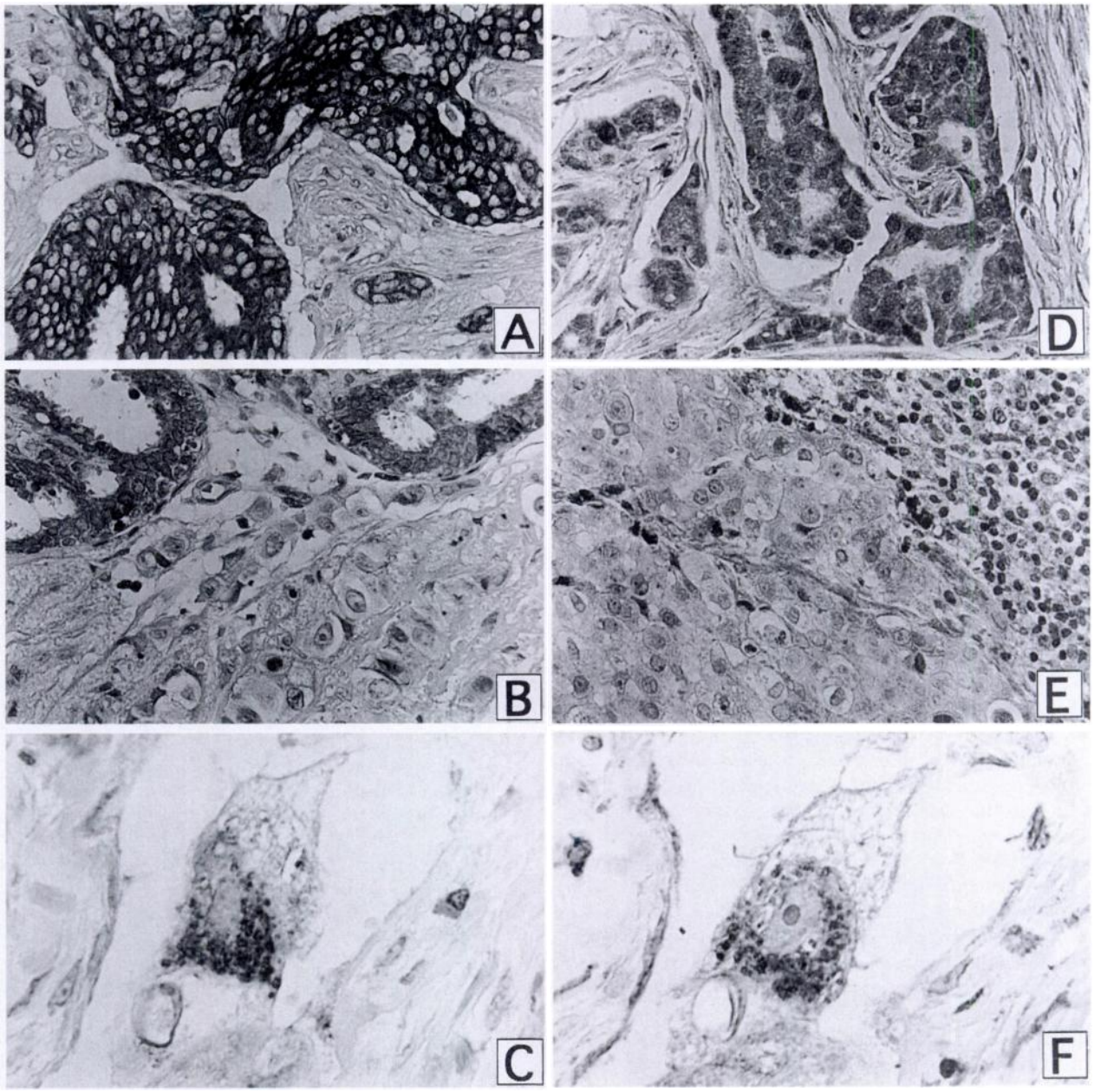

Fig. 2 Immunohistochemical analysis of Bax and Bcl-2 in human breast cancers. Representative light photomicrographs are shown for tumors immunostained using antisera specific for either Bax (*A*, *B*, and C) or Bcl-2 (*D*, *E*, and *F*) and a diaminobenzidine colorimetric detection method. Nuclear counterstaining was performed with hematoxylin. Shown are examples of tumors that contain invasive tumor cells that are mostly Bcl-2 or Bax positive (*A* and *D*) *versus* mostly Bcl-2 or Bax negative (*B* and *E*). In the examples where the infiltrating tumor cells are mostly negative, note the presence in *B* of residual normal epithelium or carcinoma *in situ* which is Bax immunopositive (*upper left corner*), thus serving as internal positive controls and Bcl-2 immunopositive infiltrating lymphocytes in *E* (*upper right corner*; ×400). The lower panels (*C* and *F*) show representative examples of the intracellular locations of Bcl-2 and Bax at higher magnification (×1000).

shows some representative results. Variable percentages of the malignant invasive cells immunostained for Bax and Bcl-2. In some cases, nearly all of the invasive tumor cells were immunopositive for Bax or Bcl-2 (Fig. 2, *A* and *D*), whereas in others, the invasive cells were nearly all negative (Fig. 2, *B* and *E*). In addition to the invasive tumor cells, Bax and Bcl-2 immunostaining were usually found in co-existing carcinoma *in situ*, residual normal or metaplastic breast epithelium, infiltrating lymphocytes, and peripheral nerves. These nonmalignant Bax and Bcl-2-immunopositive cells served as an internal positive control for verifying adequate specimen preservation in cases where the invasive component of the tumors was negative (see below). Stromal fibroblasts were negative for Bax and Bcl-2.

The specificity of these immunostaining results was con-

firmed by use of preimmune serum as well as Bax and Bcl-2 antiserum that had been preadsorbed with specific peptide antigens, both of which resulted in essentially no immunoreactivity in normal or neoplastic breast epithelial cells (data not shown). Moreover, comparisons of immunostaining and immunoblot results revealed a generally good correlation between the percentage of invasive tumor cells with Bax immunopositivity and the intensity of the p21-Bax band, thus further suggesting that the anti-Bax antibody reagent employed here is specific (see the Fig. 1 legend for details).

The Bax and Bcl-2 immunoreactivity were invariably present in a cytosolic location at the light-microscopic level, sometimes with a granular appearance suggestive of association with intracellular organelles and sometimes with perinuclear accentuation. Fig. 2, *C* and *F*, show examples of Bax and Bcl-2 immunostaining, respectively, in sequential sections through a tumor cell at high power magnification, illustrating punctuate immunostaining in association largely with perinuclear organelles. Given that several studies have documented the association of Bcl-2 protein and related proteins with mitochondrial membranes, as well as to some extent with the nuclear envelope and parts of the endoplasmic reticulum (34–40), these immunolocalization data provide further evidence of the specificity of our antisera for detection of the Bcl-2 and Bax proteins in normal and malignant mammary epithelial cells.

Comparisons of Bax and Bcl-2 Immunostaining with p53 in Breast Cancers. Immunostaining for Bax was successful in 131 of the 135 p53-immunopositive cases and 14 of 14 of the p53-negative cases (see "Materials and Methods"). Bax immunopositivity was noted in 95% of these 145 carcinomas, with the percentage of Bax-immunopositive tumor cells ranging from ~0 to 100% (median, 60%; Fig. 3*A*). Immunohistochemical analysis of Bcl-2 was successfully performed on 135 of 139 p53-immunopositive and 14 of 14 of the p53-negative cases. Bcl-2 immunopositivity ranged from 0 to 100% in 149 invasive carcinomas (median, 35%; Fig. 3*B*). When the p53-immunopositive (n = 135) and p53-immunonegative (n = 14) subgroups were compared, the median Bcl-2 immunopositivity was 20% for p53-positive tumors as compared to 85% for the p53-negative cases (P = 0.004). Bax immunopositivity was not different in the p53-positive and -negative tumor groups.

Among the p53-positive tumors, the percentage of invasive tumor cells with nuclear p53 immunoreactivity ranged from 0.5 to 95%, with a median of 5%. In these p53-positive cases, the percentages of Bax-, Bcl-2-, and p53-immunopositive tumor cells were compared by simple correlation analysis, as continuous variables. As summarized in Fig. 4, the percentage of Bax-immunopositive tumor cells was not significantly associated with the percentage of cells with p53 immunopositivity (r = −0.09; P = 0.30; Fig. 4*A*). In contrast, the percentage of Bcl-2-immunopositive cells in p53-positive tumors was inversely correlated with the percentage of p53 immunopositivity (r = −0.44; P < 0.001; Fig. 4*B*). The percentage of Bax-immunopositive tumor cells, however, was significantly associated with the percentage of Bcl-2-immunostained cells (r = 0.27; P = 0.002; Fig. 4*C*). Visual inspection of the data (Fig. 3), however, indicates that there exists a great deal of variability in the case-to-case relationships between p53 and Bcl-2 and between Bcl-2 and Bax. Furthermore, the relationships may not be linear, in terms of relative percentages of immunopositive tumor cells.

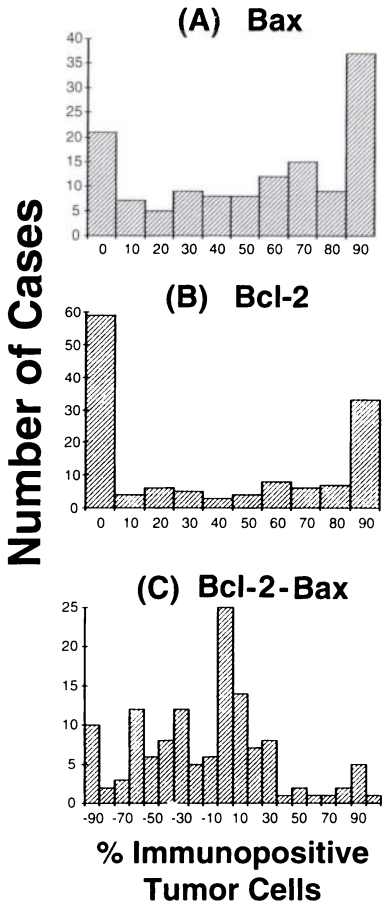

Fig. 3 Distribution of Bax and Bcl-2 in breast cancer tumors. The percentages of Bcl-2 (*A*, n = 145), Bax (*B*, n = 149), and Bcl-2-Bax (*C*) immunopositive tumor cells are shown for all patient specimens, both p53 positive and p53 negative, analyzed in this study.

Correlations of Bax and Bcl-2 with Other Tumor Characteristics and Patient Survival in p53-immunopositive Cases. The Bax and Bcl-2 immunostaining results were compared with other tumor characteristics often associated with clinical outcome and with patient survival data (Table 1). For these correlations, only the 135 p53-immunopositive cases were used because of the small size of the p53-negative group. When examined as a continuous variable, the percentage of Bax-positive invasive tumor cells did not correlate with histological grade, lymph node status, T stage, or ER status. The percentage of Bax-immunopositive tumor cells, however, was positively correlated with *erbB*-2 immunopositivity (P = 0.02). Consistent with previous reports (18–21), increased Bcl-2 expression (as a continuous variable) correlated with ER positivity (P = 0.03), low grade histology (1 and 2 *versus* 3; P = 0.03), and low T stage (P = 0.02; Table 1).

In univariate analysis, low Bcl-2 was associated with shortened DFS and OS in lymph node-positive patients (P = 0.02 and 0.004, respectively). Bcl-2, however, did not predict the

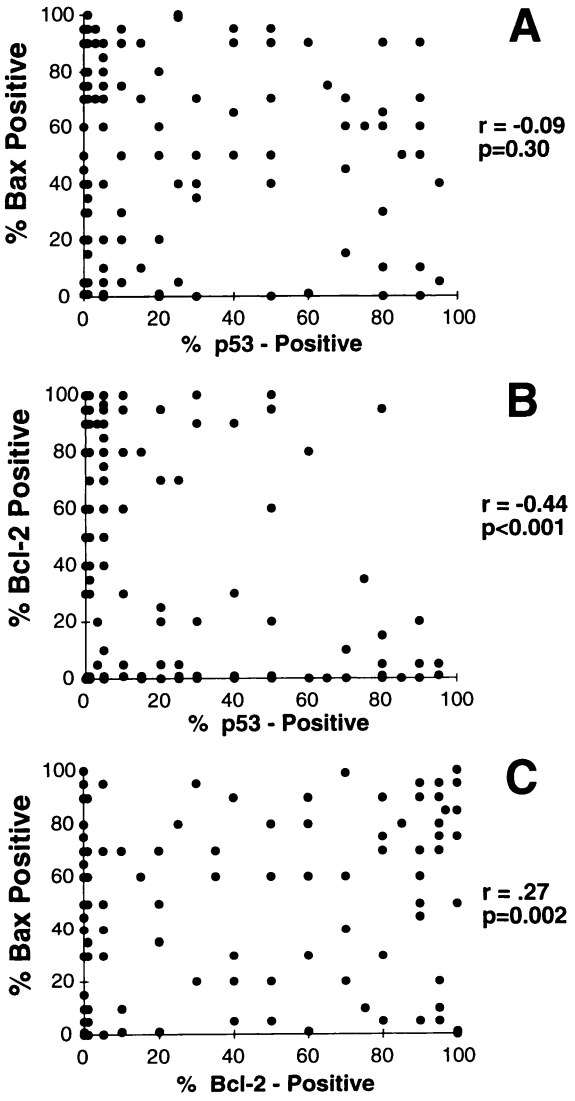

Fig. 4 Comparisons of the percentages of Bax, Bcl-2, and p53 immunopositive tumor cells. *A*, percentage of Bax-positive cells *versus* percentage of p53-positive cells; *B*, percentage of Bcl-2-positive cells *versus* percentage of p53-positive cells; *C*, percentage of Bax-positive cells *versus* percentage of Bcl-2-positive invasive tumor cells. Statistical significance was determined by Pearson correlation (r and P values are indicated).

outcome in node-negative patients. Using univariate analysis, Bax failed to predict the outcome in either node-negative or node-positive patients. Because the relative amounts of Bcl-2 and Bax can be a determinant of the relative sensitivity of tumor cells to apoptotic stimuli (6), the difference between the percentages of Bcl-2- and Bax-immunopositive tumor cells (*i.e.*, percentage of Bcl-2-positive − percentage of Bax-positive) was compared with outcome data. Note that in instances where the percentage of Bax-positive tumor cells exceeds the percentage of Bcl-2-positive cells, a negative value is obtained. Also, when the percentages of Bcl-2- and Bax-positive tumor cells are the same, the value is 0. Using this approach for simultaneously examining Bcl-2 and Bax, higher values for (Bcl-2 − Bax) were associated with longer DFS ($P = 0.04$) and OS ($P = 0.01$), but only for node-positive patients.

A Cox proportional hazards model that included T stage and histological grade was used in a multivariate assessment of the ability of Bax and Bcl-2 to predict outcome in p53-immunopositive cases (see Table 2). The LR can be used to compare results, because the same tumors and the same covariates (T stage and grade) were used in each model. Bcl-2 was significantly associated with a longer DFS (model 1, $P = 0.04$) and OS ($P = 005$) in node-positive but not in node-negative patients. Conversely, Bax was not significantly correlated with either DFS or OS in women with either node-negative or node-positive disease. When both Bax and Bcl-2 were included in the model, the signs of the coefficients were opposite, suggesting that a single term, Bcl-2 − Bax, could be used to summarize the predictive contribution of both variables. The results, shown as model 3, suggest that prediction is slightly improved by adding Bcl-2 information to that for Bax in the node-negative subset of patients tumors, as indicated by the slight increases in LR from 7.98 (model 2) to 8.32 (model 3) for DFS and from 11.52 to 12.41 for OS. No benefit was obtained, however, by adding Bax data to Bcl-2 information in node-positive cases (decreases in LR for both DFS and OS; model 1 *versus* model 3 in Table 2). It is important to note, however, that these correlations with clinical outcome should be viewed as highly preliminary, given the small number of cases and the nonuniformity of patient treatment.

DISCUSSION

Several independent reports have documented that Bcl-2 is expressed in about two-thirds of adenocarcinomas of the breast and is associated with ER positivity, low histological grade, absence of p53 mutations, and low levels of *erb*B-2 expression (18–21). In light of the recent publication by Silvestrini *et al.* (18), which suggested that Bcl-2 and p53 expression are strongly associated and that Bcl-2 fails to maintain prognostic significance in the presence of p53 expression, we sought to evaluate predominantly p53-positive breast cancers for Bcl-2 expression. We further used antisera against Bax, a pro-apoptotic protein that can heterodimerize with Bcl-2. Because the ratio of Bax to Bcl-2 protein has been shown to regulate the relative sensitivity of cells to apoptotic stimuli (6), we also performed correlations in which Bax and Bcl-2 data were considered together (*i.e.*, percentage of Bcl-2 − percentage of Bax).

In contrast to the previous report by Silvestrini *et al.* (18) that involved exclusively node-negative patients, the multivariate analysis of p53-immunopositive breast cancers described here indicates that Bcl-2 can provide independent prognostic information for node-positive patients. Moreover, although highly preliminary given the small number of patients analyzed and the nonuniformity of treatment, it appears that Bax and Bcl-2 together (expressed as Bcl-2 − Bax) are marginally helpful in predicting DFS in node-negative patients with p53-positive tumors. The clinical significance and biological ramifications of these latter results are unclear but probably relate to the ability of the Bcl-2 and Bax proteins to physically interact by

Table 1 Relations between Bcl-2, Bax, and other factors in p53-immunopositive breast cancers

Factor	Bcl-2			Bax			Bcl-2-Bax		
	No. of tumors[a]	% positive (mean ± SD)	P^b	No. of tumors	% positive (mean ± SD)	P	No. of tumors	% positive (mean ± SD)	P
Grade									
1 or 2	53	50 ± 41	0.03	50	53 ± 35	NS	50	0.1 ± 44	0.002
3	82	32 ± 38		81	57 ± 33		81	−24 ± 43	
ER status									
Negative	72	27 ± 36	0.03	69	50 ± 33	NS	69	−22 ± 48	0.05
Positive	48	58 ± 39		47	64 ± 32		47	−6 ± 34	
Positive nodes									
0	53	42 ± 41	NS	53	49 ± 35	NS	53	−7 ± 51	NS
1–3	35	45 ± 40		34	60 ± 31		34	−14 ± 39	
≥4	32	25 ± 36		30	57 ± 32		30	−31 ± 36	
T stage									
1	37	48 ± 39	0.02	36	54 ± 36	NS	36	−6 ± 45	NS
2	69	41 ± 42		68	56 ± 33		68	−14 ± 43	
3	11	12 ± 28		10	42 ± 31		10	−28 ± 40	
4	16	22 ± 30		15	60 ± 35		15	−36 ± 52	
DFS[c]									
LN-negative	53		NS	53		NS	53		NS
LN-positive	67		0.02	64			64		0.04
OS[c]									
LN-negative	53		NS	53		NS	53		NS
LN-positive	67		0.004	64			64		0.01

[a] Some cases were omitted because of missing values.

[b] Continuous variables, P based on two sample t test, statistical significance at $P \leq 0.05$.

[c] Univariate analysis tests Bcl-2 (or Bax or Bcl-2-Bax) as a predictor of DFS (or OS) in lymph node (LN)-negative and in LN-positive tumors separately.

forming heterodimers and to the opposing effects that these proteins have on cell life and death.

Our comparison of p53-negative and -positive breast cancers confirms the observations of others (18–21), who have showed that p53-immunonegative tumors expressed Bcl-2 at much higher levels than p53-positive tumors (median Bcl-2 expression was 85% in p53-negative cases as compared to 20% in p53-positive cases in this study). Although the spectrum of mutations that can occur in the p53 gene is broad and the functional alterations in mutant versions of p53 are not always predictable by immunopositivity (41), these findings raise the possibility that Bcl-2 is up-regulated rather than down-regulated by WT p53 in breast cancers, inasmuch as p53 immunopositivity is generally a reflection of mutations that lead to stabilization of the p53 protein and given that mutant p53 can usually act as a dominant inhibitor of WT p53 (41). Consistent with this idea, transfection studies involving a human breast cancer line have shown that mutant p53 can result in down-regulation of Bcl-2 expression (17). Alternatively, the negative correlation between p53 immunopositivity and Bcl-2 may be merely coincidental, reflecting perhaps progression of these tumors to a more deregulated state where Bcl-2 is no longer required for maintenance of cell survival.

Because of its ability to promote cell death, Bax would be expected to function essentially as a tumor suppressor gene, with reductions in Bax providing tumor cells with a selective survival advantage and thus contributing to their expansion. In this regard, the invasive component of many of the tumors examined here contained Bax-immunonegative cells. The molecular basis for heterogeneity in Bax expression observed in the tumors analyzed here is unknown. Benign breast epithelium normally expresses both Bax and Bcl-2 (22, 42), the levels of which are probably subject to regulation by estrogen and other factors (43). Interestingly, Bax immunostaining was independent of most other tumor markers (p53 and ER) and clinical characteristics (T stage, lymph node status, and histological grade). The *Bax* gene has been mapped to chromosome 19q13.3, but this region is not frequently altered in breast cancers, and thus deletions or other structural alterations to the *Bax* gene are unlikely to explain the changes in Bax expression seen in many breast cancers (23, 44, 45). Bax immunostaining, however, was associated with higher percentages of c-*erb*B2-positive tumor cells, raising the possibility that *erb*B2 may influence expression of Bax in breast cancers.

Unlike Bcl-2, differences in Bax expression were not observed between p53-negative and -positive invasive ductal carcinomas, although the numbers of the latter group were much larger. The percentages of Bax- and p53-immunopositive tumor cells also were not correlated when examining the data as continuous variables and including all cases, both p53 positive and negative (Fig. 3*A*). The *Bax* gene promoter contains p53 binding sites and can be transactivated by p53 in some cell lines (14–16, 46). Our data, however, suggest that loss or alteration of p53 function, by itself, is insufficient to result in diminished Bax expression, suggesting that additional genetic or epigenetic events are required. One caveat that always exists when interpreting p53 immunostaining results, however, is that several mechanisms for functional inactivation of p53 have been described, whereas p53 immunopositivity is generally associated only with missense mutations of the *p53* gene (41). Moreover,

Table 2 Comparison of various Cox models that incorporate Bax and Bcl-2 immunostaining data for outcome analysis[a]

	DFS				OS			
	LN− (N = 50, m = 16)		LN+ (N = 63, m = 39)		LN− (N = 53, d = 19)		LN+ (N = 64, d = 41)	
Factor	RR	P	RR	P	RR	P	RR	P
Model 1								
T stage	2.22	0.04	1.32	0.16	2.43	0.02	1.08	0.69
Grade	0.71	0.57	0.64	0.18	0.29	0.04	0.73	0.32
Bcl-2[b]	0.99	0.68	0.99	0.04	0.99	0.54	0.99	0.005
LR+	4.31		10.15		9.08		10.91	
Model 2								
T stage	2.76	0.01	1.44	0.05	3.03	0.006	1.22	0.27
Grade	0.66	0.45	0.62	0.15	0.30	0.02	0.70	0.26
Bax[b]	1.02	0.07	0.99	0.43	1.01	0.11	0.99	0.36
LR+	7.98		5.95		11.52		3.01	
Model 3								
T stage	2.65	0.02	1.36	0.16	2.88	0.008	1.12	0.57
Grade	0.39	0.15	0.68	0.18	0.17	0.01	0.78	0.43
(Bcl-2 − Bax)[b]	0.986	0.05	0.99	0.12	0.99	0.06	0.99	0.03
LR+	8.32		7.77		12.41		7.25	

LR, likelihood ratio. The LR can be used as a measure of the agreement between predicted and observed survival, with larger values indicating better agreement. Differences in LR less than 2 are not statistically significant; N, number of patients; m, number of patients with metastases; d, number of deaths; RR, relative risk (value indicates increase in risk of DFS (or OS) per unit change in a factor).

[a] All factors were used as continuous variables. T stage was coded as 1, 2, 3, and 4; Grade as 1, 2, and 3; Bcl-2 and Bax as percentage of positive cells; (Bcl-2 − Bax) as difference in percentages.

[b] Continuous variable.

our data do not exclude an important role for p53 in inducing increases in Bax expression above basal levels in response to DNA-damaging drugs or radiation, because genotoxic stress has been shown to up-regulate Bax expression in a p53-dependent manner in some tumor cell lines (46). In this regard, it should be noted that the breast cancers used in this study were all primary lesions, which were excised prior to chemotherapy or radiation exposure.

Interestingly, Bax and Bcl-2 protein levels appeared to be co-regulated to some extent in the p53-immunopositive breast adenocarcinomas evaluated here (P = 0.006), consistent with a previous report (22). The molecular mechanisms responsible for the regulation of Bax and Bcl-2 protein levels in these tumors remain to be determined but could entail both transcriptional and posttranscriptional mechanisms. With regards to the latter, for example, Bcl-2 has been shown to prolong the half-life of the Bax protein in some cell lines, presumably at least in part because of the ability of Bcl-2 to heterodimerize with Bax (47). Of potential relevance to transcriptional mechanisms, estrogen can up-regulate Bcl-2 expression in the MCF-7 breast cancer line (43). This observation in MCF7 cells is supported by the data presented here and elsewhere (18–21, 48, 49) showing a significant association between Bcl-2 expression and ER positivity. However, in the studies of MCF7 cells, estrogen did not modulate Bax expression. Similarly, we observed that Bax immunostaining was independent of ER status in the tumors evaluated here. Thus, co-regulation by estrogen probably cannot explain the correlation seen here between Bax and Bcl-2. Nevertheless, we hypothesize that loss of ER during breast cancer development may be an important contributor to the reduced expression of Bcl-2 seen in many of the p53-positive carcinomas evaluated here. It remains to be determined how the simultaneous loss of ER and the development p53 mutations affect expression of the *Bcl-2* gene, given that both estrogens and p53 can regulate expression of this antiapoptotic gene in breast cancer cell lines (17, 43).

Although Bax immunostaining was not significantly associated with outcome in this study of women who lacked evidence of metastatic disease, Bax immunopositivity was associated with better outcome in a previous study of breast cancer patients with metastatic disease who were treated with combination chemotherapy (22). In this regard, the Bcl-2:Bax ratio has been shown to be an important determinant of the relative sensitivity of tumor cells to apoptosis induced by numerous stimuli, including anticancer drugs (reviewed in Refs. 3–5). Gene transfer-mediated elevations in Bcl-2 in MCF-7 cells, for example, have been shown to promote resistance *in vitro* to Adriamycin (43). Conversely, higher endogenous levels of Bax and gene transfer-mediated overexpression of Bax in human breast cancer lines have been correlated with increased sensitivity to induction of apoptosis by growth factor deprivation, anti-Fas antibodies, and chemotherapeutic drugs (50, 51). For this reason, one might predict that tumors with low Bcl-2 and high Bax would be expected to exhibit better responses to therapy. The patient subset studied here, however, does not adequately test this hypothesis, because therapy was not controlled. Nevertheless, the correlation between Bcl-2 immunostaining and longer survival commonly seen in breast cancer patients suggests that examination of only one member of the Bcl-2 protein family may be inadequate for gaining a complete understanding of mechanisms that lead to dysregulation of programmed cell death during oncogenesis and that contribute to intrinsic chemoresistance in cancers (reviewed in Refs. 52). Further studies of the expression of Bax, Bcl-2, and other

members of the *Bcl-2* gene family in a randomized trial setting involving larger sets of patients that are controlled for therapy will presumably be required, therefore, to fully elucidate the prognostic relevance of these apoptosis-regulating genes for women with adenocarcinoma of the breast.

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