The Prognostic Significance of Bcl-2 and p53 Expression in Ovarian Carcinoma

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ABSTRACT

Advanced ovarian cancer is characterized by poor prognosis and the development of resistance to chemotherapy. We have found that Bcl-2 and p53, two proteins implicated in the control of apoptosis, are differentially expressed in the ovarian cell line A2780 and its cisplatin-resistant variant 2780CP, with the resistant line overexpressing both proteins. Transfection of the A2780 cells with a Bcl-2- or p53-expressing plasmid increases resistance to various drugs, including cisplatin, suggesting that Bcl-2 and p53 expression may influence the sensitivity of ovarian cancer cell lines to chemotherapy. Expression of these two proteins in vivo was determined by immunohistochemical staining of ovarian tumor biopsies from 70 patients. We found that Bcl-2 and p53 were expressed in 57 and 61% of specimens examined, respectively. Both p53 and Bcl-2 were found to be independent prognostic indicators of survival in ovarian cancer. Survival was poorer in patients with tumors expressing high levels of p53, whereas expression of Bcl-2 was associated with improved survival.

INTRODUCTION

The efficacy of cancer chemotherapy is restricted by the ability of tumors to resist or develop resistance to treatment. Ovarian cancer shows high response rates to first-line chemotherapy but is characterized by recurrence and the development of resistance to chemotherapy. Therefore, prognosis is poor, with only a minority of patients surviving 5 years. Resistance to chemotherapy has been associated with decreased susceptibility to apoptosis (1, 2), raising the possibility that cell death determinants may influence the outcome of treatment.

The bcl-2 gene, the first negative regulator of cell death to be identified, was discovered through the t(14:18) translocation, which frequently occurs in B-cell lymphomas (3). The t(14:18) translocation juxtaposes bcl-2 with the immunoglobulin heavy chain locus, resulting in deregulation of expression (4). The mechanism of action of the Bcl-2 protein has not been fully defined but may involve oxidative phosphorylation and/or mitochondrial electron and metabolite transport (5), and its main effect is to prolong cell survival by avoidance of apoptosis (6–8).

Stem cells in epithelia, neurons, and memory B cells all express Bcl-2. In epithelial tissues, which are continually renewed, Bcl-2 expression is seen in the basal layers of the epithelium but is lost as cells approach the surface of the epithelium prior to undergoing apoptosis (9). Bcl-2 is also expressed in glandular cells, such as those in the female breast, in which regulation of hyperplasia and involution is controlled by hormones and growth factors (10).

The deregulation of expression of Bcl-2 in neoplastic tissues is of interest for two reasons. First, it may be that inappropriate expression of Bcl-2 is involved in neoplastic transformation, and second, expression of Bcl-2 by tumors may confer resistance to chemotherapy by enabling cells to avoid apoptosis. Transfection of bcl-2 into NIH3T3 cells induces tumorigenicity of the transfected cells when they are injected into mice (11). Similarly, transgenic mice with deregulated bcl-2 gene develop follicular hyperplasia with extended B-cell survival, which progresses to diffuse, large cell lymphoma (12). Experiments with hematopoietic cells expressing different levels of Bcl-2 or transfection of bcl-2 into cell lines show that Bcl-2 expression confers resistance to a variety of chemotherapeutic agents (including platinum) in vitro (6, 7, 13).

Bcl-2 expression has been demonstrated in solid tumors, including non-small cell lung (8), prostate (14), colon (15), and breast (10, 16, 17). The significance of Bcl-2 expression remains uncertain, but paradoxically, retrospective archival studies of non-small cell lung and breast carcinomas suggest that Bcl-2 expression is associated with a survival advantage (8, 10, 17).

The p53 protein is a transcriptional activator that binds to specific DNA sequences in the control regions of genes, influencing their expression (18). This leads to expression of specific genes necessary for inhibition of cell growth or, alternatively, apoptosis (7). Alterations of p53 activity, either as a result of point mutations or deletions or due to protein stabilization in the absence of obvious genetic changes, are the most frequent abnormalities seen in human cancers (19). These alterations lead to the loss of wild-type p53 function and may thus allow uncontrolled growth of damaged cells. Indeed, accumulation of the protein has been shown to be a prognostic marker of reduced survival in breast, gastric, and non-small cell lung cancer (19, 20). Whereas the expression of wild-type p53 can induce cell death or growth arrest in a number of cell systems (21, 22), expression of a mutated form of the protein in hematopoietic cells has been shown to protect against apoptosis induced by chemotherapeutic agents (7). Furthermore, fibroblasts from Li-Fraumeni patients heterozygous for a p53 mutation are radioresistant (23), suggesting that the loss of function of p53 could have an effect similar to that of the gain of function of Bcl-2.

Given the apparent role of Bcl-2 and p53 expression in oncogenesis and resistance to chemotherapy, we decided to establish whether Bcl-2 and p53 are expressed in ovarian carcinoma and whether expression of these genes has any prognostic significance or relationship to the response to chemotherapy.

MATERIALS AND METHODS

Cell Lines, Plasmids, and Transfections. The human ovarian carcinoma cell line A2780 and its cisplatin-resistant subclone 2780CP (24) were maintained in DMEM (Life Technologies, Inc., Uxbridge, United Kingdom) supplemented with 10% FCS (Flow Laboratories, Irvine, United Kingdom).

The A2780 cell line was transfected with the plasmid pCdJ-bcl-2 (25), carrying the human bcl-2 cDNA or with a control plasmid only, using the calcium phosphate technique. Stable transfectants resistant to the selective marker genetin G418 (Life Technologies) were obtained. The A2780 cells were also cotransfected with the plasmids pLTRGVal135, expressing a ts4 mutant p53 (tsp53) protein (kindly provided by Prof. M. Oren, Weizmann Institute of Science, Rehovot, Israel; Ref. 22), and pSV2gpt, conferring resistance to mycophenolic acid, or they were cotransfected with the control plasmid pSV2gpt.

The abbreviations used are: ts, temperature sensitive; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IC50, 50% inhibitory concentration; CI, confidence interval.

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**RESULTS**

Expression of Bcl-2 and p53 in the Ovarian Cell Line A2780

**Confers Resistance to Chemotherapeutic Agents.** Bcl-2 and p53 are differentially expressed in the ovarian cell lines A2780 and 2780CP, with the cisplatin-resistant 2780CP cells overexpressing both proteins (Fig. 1A). To determine whether Bcl-2 and p53 directly contribute to the development of resistance in vitro, A2780 cells were transfected with a Bcl-2- or tsp53-expressing plasmid. Western blot analysis was used to verify the expression of the genes in the isolated transfectants (Fig. 1B).

The Bcl-2 and p53 clones were subsequently tested for response to...
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Fig. 1. A. Western blots showing overexpression of Bcl-2 (left panel) and p53 (right panel) in the resistant ovarian cell line 2780CP compared with the parental A2780 line. One hundred mg protein were analyzed for Bcl-2 and p53 expression using the monoclonal antibodies bcl-2:124 and pAb1/801 (29), respectively. Probing with proliferating cell nuclear antigen antibody (PC10; DAKO) verified equal protein loading and transfer efficiency (data not shown). B. Western blots showing overexpression of Bcl-2 (left panel) and tsp53 (right panel) in Bcl-2 and tsp53 A2780 transfectants, respectively. The antibodies used were the monoclonal bcl-2:124 and p53 polyclonal antibody SAPO, which recognizes both human and mouse p53. C. Cytotoxicity curves from a typical MTT assay showing the effect of cisplatin on the viability (A550) of the A2780 ovarian cell line, a Bcl-2 transfectant (A2780bcl-2/Cl.14), a tsp53 transfectant (A2780tps/p53/Cl.11), and the resistant variant 2780CP. Bars, 3 SD. The A550 for the untreated cells were 0.50 ± 0.05, 0.49 ± 0.02, 0.48 ± 0.03, and 0.46 ± 0.03, respectively. The shift of the curve to the right is characteristic of an increase in viability.

cisplatin-induced cytotoxicity by the MTT colorimetric assay. Representative cytotoxicity curves for one of the Bcl-2 transfectants (A2780bcl-2/Cl.10), one of the p53 transfectants (A2780tps/p53/Cl.1) induced to express mutant p53, the parental A2780, and the 2780CP (cisplatin-resistant) cell lines are shown in Fig. 1C. The IC_{50} values were 4.0 \times 10^{-6} \text{ M} for the parental line, 5.1 \times 10^{-5} \text{ M} for the resistant 2780CP line, 1.4 \times 10^{-5} \text{ M} for the bcl-2-transfectant clone, and 7.5 \times 10^{-6} \text{ M} for the p53 transfectant. A two-way ANOVA was carried out to assess the relative resistance of the different cell lines to cis-platinum. A significant difference exists between the four dose-response curves (F = 43.46; P < 0.0001). To identify the source of this difference, the Student-Newman-Keuls method (32) was used and...
found no significant difference between the dose-response curves for the A2780Bcl-2 and A2780ts/p53 cell lines. However, these dose-response curves were significantly different from those of the A2780 and 2780CP cell lines. Bcl-2 conferred a 2.3–3.5-fold and mutated p53 conferred a 1.7–2.4-fold resistance to platinum in the A2780 ovarian cell line. However, the levels of resistance in the Bcl-2 and p53 transfectants were lower than those for the 2780CP cell line (12.2-fold resistance compared with that of A2780), suggesting that additional factors contribute to the development of resistance.

Bcl-2 and p53 were also found to induce similar levels of resistance (2–4-fold) to other chemotherapeutic agents, such as lobaplatin, Adriamycin, and etoposide. Thus, Bcl-2 and p53 increased the IC₅₀ value from 2.7 × 10⁻⁶ to 1.2 × 10⁻⁵ and 6.8 × 10⁻⁶ M, respectively. Similarly, resistance to Adriamycin increased from 3.3 × 10⁻⁶ to 6.9 × 10⁻⁶ M (Bcl-2) and 4.2 × 10⁻⁶ M (p53), and resistance to etoposide increased from 1.2 × 10⁻⁶ to 4.8 × 10⁻⁶ M (Bcl-2) and 3.0 × 10⁻⁶ M (p53).

**Immunostaining for the Bcl-2 Protein.** Of the 70 specimens examined, 40 (57%) stained positive for the Bcl-2 protein [17 (24%) of 70, ≥75%; and 23 (33%) of 70, <75%], and 30 (43%) were negative. Positive staining was confined to the cytoplasm of carcinoma cells (Fig. 2, A and B). The pattern of staining showed great variation between specimens. Some sections showed labeling of the vast majority of cells (Fig. 2A), whereas in others, only small areas of tumor were found to be positive for the Bcl-2 protein (Fig. 2B). In the majority of positive sections, the focal nature of staining was a striking feature, with positively and negatively staining areas being found in close apposition (Fig. 2B). Positive staining for the Bcl-2 protein was also seen in normal ovarian tissue in the theca interna cells of the corpus luteum, granulosa cells of ovarian follicles, and also in ovarian stroma.

**Immunostaining for the p53 Protein.** Forty-three (61%) of the specimens showed positive staining for p53 protein [28 (40%) of 70, ≥75%; and 15 (21%) of 70, <75%], and 27 (39%) were negative.
Staining in carcinoma cells was nuclear, and, when positive, virtually all cells were labeled (Fig. 2, C and D). No staining was observed in normal ovarian tissue.

Survival and Expression of Bcl-2 and p53. Overall survival for these patients was poor, with 39% survival at 2 years (95% CI, 27–51%) and 12% survival at 5 years (95% CI, 4–20%). Log-rank univariate analysis was carried out as shown in Table 2. Performance status (P < 0.03), residual disease (P < 0.0001), creatinine clearance (P < 0.0001), and histological type (P < 0.0001) were identified as significant prognostic factors. No significant differences in survival between negative, weakly positive, and strongly positive tumors for either Bcl-2 (P = 0.1) or p53 (P = 0.45) were found.

Cox regression analysis was carried out on all 70 patients (Table 3). Variables considered were trial, treatment, performance status, residual disease, stage, histological grade and type, albumin level, creatinine clearance, time from primary surgery to chemotherapy, age, menopausal status, Bcl-2 expression, and p53 expression. The first factor to be selected was histological type (clear cell or not clear cell), followed by creatinine clearance at entry to the trial (≤60 or >60), residual disease (≤2 or >2 cm), Bcl-2 (≥75 or <75%), and finally, p53 (≥75% or <75%). No other variables enter the model. These five factors were shown to be independently prognostic by excluding each in turn from the set of possible prognostic variables and allowing those remaining to compete to enter the model in its place. Histological type, creatinine clearance, and residual disease were widely accepted as prognostic factors. To demonstrate the effect of p53, survival curves were obtained from the model containing histological type, creatinine clearance, residual disease, and p53 expression (Fig. 3A). Patients with <75% of cells stained positive have a better prognosis than those with ≥75%. The size of this effect for patients with the worst prognosis is small. However, having <75% of cells positively stained improves survival at 2 years by 10% (46 versus 36%) for the intermediate prognostic group and 6% (74 versus 68%) for the best prognostic group. Similarly, predicted survival curves were obtained from the model containing histological type, creatinine clearance, residual disease, and Bcl-2, only to demonstrate the prognostic effect of Bcl-2 (Fig. 3B). Patients with ≥75% of cells positively stained have the better prognosis, but again, the effect is small in patients with the worst prognosis. In the intermediate prognostic group, having ≥75% of cells positively stained improves survival at 2 years by 13% (41 versus 28%), and for the best prognostic group, an improvement of 9% (74 versus 65%) at 2 years is seen. Finally, predicted survival curves for the full model with all five factors were obtained (Fig. 3C).

Response to Chemotherapy. There was no significant association between overall response to chemotherapy (complete or partial response versus static or progressive disease) and p53 (P = 0.69) or Bcl-2 expression (P = 0.47).

DISCUSSION

This study reports on a subset of patients from two completed Phase III randomized trials. We have found that Bcl-2 and p53 are widely expressed in epithelial ovarian tumors (57 and 61%, respectively). The proportion of tumors found to be positive for p53 is concordant with previously published work (33–35), whereas no data are available for Bcl-2 expression. Bcl-2 but not p53 staining showed heterogeneity in the majority of positive cases. No relationship between the expression of these two proteins was found. It is accepted that overexpression of the p53 protein as detected by immunohistochemistry is usually due to an underlying mutation of the p53 gene, leading to the expression of an abnormal and stabilized protein (36). Loss of p53 function, together with stabilization of the protein, however, may be caused by other mechanisms, such as interaction of the wild-type p53 protein with other cellular or viral proteins (37). Thus, although immunohistochemical detection of p53 does not necessarily indicate expression of a mutated protein, it may be a useful marker for the presence of a functionally abnormal p53 protein (38).
Staining for Bcl-2 was also noted in normal ovarian tissue, e.g., in ovarian stroma, which is in accordance with a role for Bcl-2 in the survival of epithelial stem cells (9). Bcl-2 is also expressed in granulosa and theca cells. These cells are under cyclical control by estrogen and undergo rapid proliferation during each menstrual cycle before dying off, presumably by programmed cell death, should conception fail to occur after ovulation. Expression of Bcl-2 in these circumstances is similar to that reported in glandular tissues of the breast and would support the hypothesis of an association between Bcl-2 expression and estrogen production (10). No expression of p53 was found in normal ovarian tissue.

The Cox regression model identifies histological type, residual disease, creatinine clearance, p53 expression, and Bcl-2 expression as significant prognostic variables. Histological type, residual disease, and creatinine clearance are the most important, with the prediction of good, intermediate, and poor prognostic groups possible on the basis of these three factors alone. The risk of death for an individual with clear cell carcinoma is 25-fold higher than that of an individual with other histological types. Although the CI is wide, the increased risk of death is at least 8-fold. Similarly, having residual disease of >2 cm increases the risk of death by at least 47%, and having creatinine clearance of ≤60 ml/min (i.e., being fit for platinum at full dose) reduces the risk of death by at least 40%.

p53 and Bcl-2 expression are weaker prognostic variables. The risk of death for individuals with ≥75% of cells staining positive for p53 is 32% greater than for those with <75% of cells positively stained. The CI is wide, however, and shows the risk to be between 1 and 74%. Similarly, our data show that the risk of death for patients with ≥75% of cells positively stained for Bcl-2 is 30% less than for those with
ovarian cell line A2780 confers relative resistance to cisplatin, the most active drug for the treatment of ovarian cancer, as well as other chemotherapeutic agents. We hypothesized that tumors that stained positively for p53 and Bcl-2 would be clinically resistant to platinum and might have a poor prognosis, given the important role of platinum in the management of ovarian cancer.

This seems to be the case for p53, which, in our study, was identified as an independent marker of poor prognosis. Our findings contradict previous work, which has failed to find such an association, in either early or late stage disease (33, 34). Interestingly, however, Bcl-2 seem to be an independent prognostic factor of improved survival. This is in agreement with previous reports in lung and breast cancer, which correlated Bcl-2 expression with a survival advantage (8, 10, 17). The power of the study was not sufficient to correlate p53 or Bcl-2 expression to the clinical response to cisplatin. However, it is possible that residual cancer following platinum treatment will be enriched in p53 and foci of Bcl-2-positive cells. In vitro, there is some evidence to suggest that certain solid tumor cell lines transfected with Bcl-2 grow more slowly (39). This could explain the apparent paradox of a gene that confers resistance to chemotherapeutic agents in vitro being associated with good prognosis.

Another plausible explanation is that other members of the overgrowing bcl-2 family, such as bax and bcl-x, may interfere with the outcome of chemotherapy. Indeed, it has been postulated that Bcl-2 may not be the critical factor for susceptibility to an apoptotic stimulus per se, but that of greater importance is the ratio of bcl-2 to bax, a gene that encodes a dominant inhibitor of Bcl-2 (40). On the other hand, the Bcl-x protein has been shown to protect against apoptosis, and its expression in many tissues (41) raises the possibility of a significant, still unknown, role in vivo.

It is unlikely that, in the clinical setting, expression of Bcl-2 or p53 by a tumor would be valuable prognostic information. However, further elucidation of the mechanisms by which these two oncogenes affect prognosis may be important in our future attempts to improve chemotherapy for and survival in ovarian carcinoma.

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