ABSTRACT

Normal or immortal epithelial cells are sensitive to a form of apoptosis, commonly referred to as anoikis, which is induced by detachment from the extracellular matrix (ECM). In contrast, development of carcinomas is associated with acquisition of cellular resistance to anoikis. However, whether human cancer cells deprived of anoikis resistance necessarily display reduced tumorigenic properties in vivo is unknown. We decided to address this question using human ovarian carcinoma cells as a model. Bcl-XL, an apoptotic factor considered to play an important role in (resistance to) anoikis, is overexpressed in ovarian cancer, and represents an unfavorable prognostic indicator for this type of human malignancy. We therefore evaluated whether Bcl-XL can be used as a tool to manipulate anoikis resistance and tumorigenicity of ovarian cancer cells. We show here that when nonmalignant ovarian epithelial cells are detached from the ECM, down-regulation of Bcl-XL and apoptotic cell death are observed, although these events do not occur in ovarian carcinoma cells. Moreover, enforced down-regulation of Bcl-XL by transfection with anti-sense cDNA in the anoikis-resistant and highly tumorigenic HEY ovarian carcinoma cell line had no impact on the viability of these cells under adherent conditions but caused significant apoptosis in response to detachment from the ECM. This change was associated with a strong inhibition of tumorigenicity of the Bcl-XL-deficient HEY cells in nude mice, both s.c. and in the peritoneal cavity. These results suggest a critical role for Bcl-XL in the maintenance of anoikis resistance in ovarian cancer cells. They also serve to establish a functional linkage between this property and the ability of human cancer cells to grow aggressively in vivo. Consequently, targeting molecular mechanisms responsible for anoikis resistance may serve as a potentially effective therapeutic strategy for the treatment of such human malignancies as ovarian cancer.

INTRODUCTION

A critical feature of carcinoma development and growth is the ability of transformed epithelial cells to survive under “anchorage-independent” (“spheroid” or three-dimensional) growth conditions, i.e., unattached to a basement membrane. Normal, or even immortalized, epithelial cells die of a form of apoptosis known as “anoikis” when forced to grow under anchorage-independent conditions in vitro (1–3) although transformed tumorigenic epithelial cells usually do not (1, 4, 5). This ability to grow in the absence of proper contacts with the ECM is thought to provide carcinoma cells with the means of surviving as multicellular spheroids in vitro and as single cells or small clumps in the bloodstream. As such, resistance to anoikis may be a major factor in acquisition of metastatic growth properties (6, 7). Likewise, the capacity of ovarian cancer cells to survive in vivo as single cells or small clumps in the ascites fluid of the peritoneal cavity (Ref. 8; i.e., what might be termed an “in vivo suspension culture”) may be a consequence of failure to undergo anoikis. The ability of various other cancers to grow as malignant effusions, e.g., gastric, breast and colon carcinomas, may also be facilitated by anoikis resistance mechanisms. Despite the potential importance of such resistance for tumor cell survival and growth, no functional link between the ability of human cancer cells to survive in the absence of the ECM and their tumor-forming capacity has been established.

We have been interested in studying some of the possible mechanisms that result in transformed epithelial cells acquiring a relative resistance to anoikis, with particular emphasis on the role of pro-oncogenic or oncogenic changes. For example, nonmalignant immortal rat intestinal epithelial cells (IEC-18) undergo massive apoptosis when plated in suspension culture or as multicellular spheroids (4). In contrast, tumorigenic transformation of IEC-18 cells by transfection of an activated, mutated ras oncogene results in this anoikis process being aborted (4). Subsequent studies suggested a role for down-regulation of a proapoptotic protein, Bak, and up-regulation of an antiapoptotic effector, Bcl-XL—both members of the Bcl-2 family of apoptosis regulators (9, 10)—in the ras oncogene-mediated protective effect on IEC-18 cells against anoikis (11, 12).

Given the possibility that Bcl-XL may contribute to anoikis resistance in some types of epithelial cells, we became interested in its possible contribution to this phenotype and to the tumorigenicity of human ovarian carcinoma cells. This interest also stemmed in part from previous studies showing that stabilization of Bcl-XL protein levels after Taxol treatment in human ovarian carcinoma cells grown as spheroids in vitro may protect against the cytotoxic effects of this drug (13). Indeed, much of the current interest in Bcl-XL with respect to cancer derives from its putative role as a possible mediator of drug resistance (6, 14–16). However, given its expression in human ovarian cancer and association with a less favorable prognosis, we reasoned that Bcl-XL may also contribute to the tumorigenic properties of ovarian carcinoma, including its ability to grow as ascites tumors.

We therefore investigated the contribution of Bcl-XL to the three-dimensional growth capacity of ovarian cancer cells as multicellular spheroids in vitro and as tumors in vivo, hypothesizing that ovarian carcinoma cells having down-regulated Bcl-XL would become susceptible to apoptosis when plated in three-dimensional culture. To do so, we studied both normal epithelial ovarian cells and ovarian carcinoma cells and used an antisense Bcl-XL cDNA transfection approach to manipulate Bcl-XL levels.

MATERIALS AND METHODS

Cell Culture. The HEY human ovarian carcinoma cell line was originally obtained from Dr. R. Buick from the Ontario Cancer Institute. The SKOV3 and CaOV3 cell lines were from the American Type Culture Collection (Rockville, MD). The normal ovarian epithelial cells were generated from patients undergoing prophylactic oophorectomies and were supplied by Dr. Ted Brown at Mount Sinai Hospital. Immunohistochemical analysis was performed, and the cells were stained positively for cytokeratins 8 and 18 to confirm epithelial origin. The cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). The growth...
medium was supplemented with 50 mg/ml penicillin-streptomycin. Cell cultures were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂.

Multicellular spheroids were prepared by the liquid overlay techniques, as described previously (17). In brief, 24-well tissue culture plates (Nunc) were coated with 0.25 ml of 1% Seaplaque Agarose (FMC) prepared from a 4% stock solution. To each well, 1 ml of 10⁵ cells was added. The 24-well plates were placed on an orbital shaker and rotated at 250 rpm for 5 min to allow the cells to come in close contact with one another.

Vector Construction and Transfection. To generate the antisense Bcl-X₉ expression vectors, the human Bcl-X₉ cDNA was inserted into the EcoR1 site in the antisense orientation. HEY cells stably expressing antisense Bcl-X₉ were generated as follows. Cells (2 × 10⁶) were transfected with 10 μg of the expression vector carrying the human Bcl-X₉ cDNA in an antisense orientation by using Lipofectin. Transfected cells were then selected in 400 μg/ml of G418. Selected clones were expanded, and down-regulation of Bcl-X₉ expression was assessed by Western blotting.

Western Blot Analysis. Cells grown in monolayer or spheroid culture were harvested and lysed with lysis buffer supplemented with 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysate was then centrifuged at 14,000 rpm for 15 min, and the postnuclear supernatant was harvested and sampled for quantitation of protein concentration, using the Bradford Dye. Twenty μg of the lysate were then mixed with 5 × SDS-PAGE sample buffer, boiled for 5 min, and subjected to electrophoresis in 12% SDS gels under reducing conditions. The separated protein samples were then electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). After blocking by 10% nonfat dried milk in Tris-buffered saline with 0.125% Tween 20 (TBS-T) for 1 h at room temperature, the membranes were blotted with an anti-Bcl-X₉ antibody (Transduction Laboratories) at a concentration of 0.25 μg/ml. After washing in TBS-T, the membrane was incubated with an antirabbit immunoglobulin: horseradish peroxidase (1:1000 dilution) for 1 h. Protein bands were detected by enhanced chemiluminescence ECL Detection System (Amersham Pharmacia Biotech).

Apoptosis Assessment: Cell Death Detection ELISA. Cells 1 × 10⁵ were plated per well in either spheroid or monolayer cultures in 24-well tissue culture plates. Forty-eight h later, cells were harvested, washed once with PBS, and assayed for the presence of nucleosomal fragments in the cytoplasm by the Cell Death Detection ELISA kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions.

Soft Agar Colony Formation (Anchorage-independent) Growth Assay. Five thousand cells were suspended in 2 ml of RPMI 1640 supplemented with 10% fetal bovine serum containing 0.3% of melted bactoagar. The resulting suspension was added to a 60-mm plate covered with a 2-ml layer of solidified 0.5% bactoagar in RPMI 1640. Cell colonies were allowed to form for 7–10 days under reducing conditions. The separated colonies were then harvested and lysed with lysis buffer supplemented with 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysate was then centrifuged at 14,000 rpm for 15 min, and the postnuclear supernatant was harvested and sampled for quantitation of protein concentration, using the Bradford Dye. Twenty μg of the lysate were then mixed with 5 × SDS-PAGE sample buffer, boiled for 5 min, and subjected to electrophoresis in 12% SDS gels under reducing conditions. The separated protein samples were then electrophoretically transferred to Immobilon-P membrane (Millipore, Bedford, MA). After blocking by 10% nonfat dried milk in Tris-buffered saline with 0.125% Tween 20 (TBS-T) for 1 h at room temperature, the membranes were blotted with an anti-Bcl-X₉ antibody (Transduction Laboratories) at a concentration of 0.25 μg/ml. After washing in TBS-T, the membrane was incubated with an antirabbit immunoglobulin: horseradish peroxidase (1:1000 dilution) for 1 h. Protein bands were detected by enhanced chemiluminescence ECL Detection System (Amersham Pharmacia Biotech).

RESULTS

Normal, Nonmalignant Ovarian Epithelial Cells Undergo Apoptosis when Plated in Three-dimensional Culture. To investigate the hypothesis that Bcl-X₉ may play a role in anoikis of ovarian epithelial cells, normal human ovarian epithelial cells were established from ovarian tissue from patients undergoing prophylactic oophorectomies. Survival of these cells when plated in monolayer or three-dimensional culture was studied using a cell death detection ELISA kit, which detects the presence of oligonucleosomes in the cytoplasm of apoptotic cells. When plated in three-dimensional culture for 48 h, the normal cells underwent a 3.6-fold increase in apoptosis compared with the monolayer cultured cells (Fig. 1A). In contrast, the HEY ovarian carcinoma cells did not undergo apoptosis when plated in three-dimensional culture (Fig. 1A). The Bcl-X₉ protein was then analyzed. Down-regulation of Bcl-X₉ protein was found when the normal cells were plated as spheroids (i.e., detached from the ECM) for 48 h (Fig. 1B). This is in marked contrast to the HEY cells, where equivalent levels of Bcl-X₉ protein were observed in the monolayer and spheroid cultures (Fig. 1B). These data therefore imply that the normal ovarian epithelial cells appear sensitive to anoikis, and this is associated with down-regulated expression of Bcl-X₉.

Resistance to Anoikis of Ovarian Carcinoma Cells Is Associated with Sustained Expression of Bcl-X₉. To ensure that independence of Bcl-X₉ expression on the presence of the ECM is not a unique property of HEY cells, two other anoikis-resistant ovarian carcinoma cell lines (CaOV3 and SKOV3) were plated in monolayer or spheroid culture for 48 h, and their Bcl-X₉ levels were analyzed (Fig. 2). The levels of Bcl-X₉ protein were adhesion independent in both cell lines, similar to that observed with the HEY cell line.

Enforced Down-Regulation of Bcl-X₉ Promotes Anoikis. To investigate the contribution of Bcl-X₉ to the resistance of human ovarian cancer cells to anoikis, we transfected the HEY ovarian carcinoma cell line with an antisense Bcl-X₉ cDNA expression vector. Three clones (designated 18, 20, and 28) were found to express significantly less Bcl-X₉ compared with the parental HEY cells or a clone transfected with vector alone (designated veccon), whereas levels of other Bcl-2 family members such as Bak, Bax, and Bad remained unchanged (Fig. 3A). In monolayer culture, there were no significant differences in the morphology or doubling times of the antisense Bcl-X₉ clones compared with the parental and vector control cells (data not shown). To determine whether enforced down-regulation of Bcl-X₉ promotes anoikis, two other anoikis-resistant ovarian carcinoma cell lines (CaOV3 and SKOV3) were plated in monolayer or spheroid culture for 48 h. Results represent the average of two independent experiments; bars, SD. B, Western blot analysis of Bcl-X₉ protein was performed in normal ovarian epithelial cells and HEY ovarian carcinoma cells plated in monolayer (ml) and spheroid (sph) culture for 48 h. The membrane was reprobed with an anti-Cdk4 antibody as a loading control.

Fig. 1. Normal ovarian epithelial cells (normal ov) are sensitive to anoikis, and this is associated with a down-regulation of Bcl-X₉ expression. A, analysis of apoptosis by a cell death ELISA in normal ovarian epithelial cells and HEY ovarian carcinoma cells plated in monolayer (ml) and spheroid (sph) culture for 48 h. Results represent the average of two independent experiments; bars, SD. B, Western blot analysis of Bcl-X₉ was performed in normal ovarian epithelial cells and HEY ovarian carcinoma cells plated in monolayer and spheroid or three-dimensional culture (3-D) for 48 h. The membrane was reprobed with an anti-Cdk4 antibody as a loading control.
regulation of Bcl-XL in these cells has an impact on anoikis, the clones described above were plated in monolayer or three-dimensional culture, and apoptosis was measured by the cell death ELISA. In three-dimensional culture, the cells transfected with antisense Bcl-XL displayed a significant increase in apoptosis, compared with the control cells, which remained viable (Fig. 3B). Under these anchorage-independent conditions, clone 28 underwent a 6.29-fold increase in apoptosis, clone 20 underwent a 5.02-fold increase, and clone 18 underwent a 2.68-fold increase compared with the parental cells. In contrast, survival of the antisense Bcl-XL clones in monolayer culture was similar to that of the control cells (data not shown). Consistent with these results, the relative competence of the antisense Bcl-XL clones to grow in soft agar was strongly inhibited (Fig. 3C). Clone 28 demonstrated 20.2% anchorage-independent growth compared with the parental cells, whereas clone 20 demonstrated 35.6%, and clone 18 demonstrated 48.8%. Taken together, these results indicate that down-regulation of Bcl-XL causes the ovarian carcinoma cells to undergo apoptosis under conditions where they are denied substrate attachment.

**Down-Regulation of Bcl-XL Inhibits Tumorigenicity.** Consistent with the anchorage-independent (spheroid) tissue culture studies, cells transfected with antisense Bcl-XL were significantly less tumorigenic *in vivo* than the respective controls, i.e., the parental cells as well as the vector control (Fig. 4A). When injected s.c. into nude mice, clones 18, 20, and 28 were 62, 83, and 95% less tumorigenic than the parental HEY cells, respectively, at day 31. When the parental cells were injected, the tumors grew to 1 cm³ in 31 days, at which time the mice had to be sacrificed. By comparison, even after day 75, 40% of the mice injected with the clone 28 cells were healthy and viable and displayed no evidence of tumor growth.

Because clinical ovarian carcinoma in advanced stages often manifests itself as an ascites growing within the peritoneal cavity, the clones and controls were also injected i.p. (i.e., orthotopically), and survival times of the mice were determined (Fig. 4B). After day 37, all of the mice injected with the parental HEY cells were dead. In contrast, even at day 120, 100% of the mice injected with clone 28 were alive. In the cases of clones 20 and 18, 60 and 40% of the mice were alive at day 120, respectively. Of note, the least tumorigenic clone, i.e., clone 28, also underwent the greatest degree of apoptosis when plated in three-dimensional culture and had the lowest soft agar colony-forming efficiency *in vitro*. Because the clones retained some degree of tumorigenicity, we investigated whether cells comprising these tumors acquired a selective growth advantage through the re-expression of Bcl-XL. The tumors were removed from the mice, and the cell lines were then reestablished in tissue culture. The cell lines were subsequently examined for their expression of Bcl-XL. As shown in Fig. 4C, we found a reexpression of Bcl-XL in the antisense Bcl-XL clones, suggesting that the loss of the inhibitory effect of the antisense Bcl-XL cDNA on Bcl-XL expression was the likely cause of tumorigenicity, and that elevated Bcl-XL expression may, therefore, likely provide a selective growth advantage for the HEY ovarian cancer cells *in vivo*.

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**Fig. 2.** Resistance to anoikis of ovarian carcinoma cells is associated with sustained expression of Bcl-XL. In addition to the HEY cell line, Western blot analysis of Bcl-XL was performed in two other anoikis-resistant ovarian carcinoma cell lines, CaOV3 and SKOV3, when plated in monolayer (ml) and in spheroid (sph) culture. The membrane was reprobed with an anti-Cdk4 antibody as a loading control.

**Fig. 3.** Enforced down-regulation of Bcl-XL promotes anoikis. A, Western blot analysis of Bcl-XL in the HEY ovarian carcinoma cells (par), HEY clones transfected with an antisense Bcl-XL expression vector (designated 28, 20, and 18), or vector alone (vecon). The membrane was reprobed with an anti-Cdk4 antibody as a loading control. To ensure specificity of the antisense approach, Western blot analysis of three of the other Bcl-2 family members, Bak, Bax, and Bad was performed. B, analysis of apoptosis by cell death ELISA in parental HEY cells (par), antisense Bcl-XL-transfected clones (18, 20, and 28), and vector control cells (vecon) under anchorage-independent conditions. Cells were plated in soft agar in triplicates, and colonies were counted after 10 days. Results are expressed as a percentage of the number of colonies obtained with the HEY parental cells and represent the average of three independent experiments; bars, SD.
cell lines were reestablished in tissue culture, and levels of Bcl-XL were analyzed. Each Bcl-XL in tumors derived from the HEY cells (determined. The number of mice used in each group was 10.

Indicated cell lines were injected i.p. into nude mice, and survival times of the mice were measured at the indicated time points. Parental, HEY cells; veccon, vector control cells; and 28, 20, and 18, antisense Bcl-XL clones. The number of mice used in each group was 5; bars, SE. This experiment was repeated twice with similar results. In B, the indicated cell lines were injected i.p. into nude mice, and survival times of the mice were determined. The number of mice used in each group was 10. C. Western blot analysis of Bcl-XL in tumors derived from the HEY cells (par), vector control cells (veccon), and antisense Bcl-XL-transfected clones (28, 20, and 18). Tumors were extracted from mice, cell lines were reestablished in tissue culture, and levels of Bcl-XL were analyzed. Each lane represents a tumor derived from an independent mouse. The membrane was reprobed with an anti-Cdk4 antibody as a loading control.

DISCUSSION

We have shown that detachment of normal human ovarian carcinoma cells from the ECM results in a significant down-regulation of the antiapoptotic protein Bcl-XL and consequently, anoikis. In the case of malignant ovarian cancer cells, similar levels of the Bcl-XL protein renders the cells resistant to anoikis upon detachment from the ECM, compared with the adherent cells where the basement membrane is intact. The possible functional significance of this finding was made evident by the demonstration that enforced down-regulation of Bcl-XL in HEY ovarian cancer cells by transfection with antisense Bcl-XL cDNA rendered the clones sensitive to anoikis and significantly decreased their tumorigenicity compared with the parental cells. Thus, our results demonstrate for the first time a link between expression of a prosurvival gene/protein and anoikis resistance in human cancer cells. Bcl-XL has no known activity in stimulating mitogenesis and is ultimately involved in cell survival. Therefore, the results would appear to be based solely on the fact that the ovarian cancer cells possess a survival advantage compared with the antisense Bcl-XL clones in that their levels of the antiapoptotic protein, Bcl-XL, are significantly higher.

Because the transfection with antisense Bcl-XL does not completely block expression of the protein but merely down-regulates it, a certain level of Bcl-XL may be necessary to confer anoikis resistance as well as result in a significant degree of tumorigenicity. We conclude that high levels of Bcl-XL and consequent resistance to anoikis are likely to be critical components of the tumorigenic phenotype in ovarian carcinoma cells. It is interesting, therefore, to note that Bcl-XL is known to be overexpressed in human ovarian carcinoma cells growing in patients, compared with adjacent normal ovarian tissue (14, 18, 19). On the basis of these finding, we speculate that Bcl-XL overexpression may contribute to anoikis resistance in ovarian carcinoma cells. This occurs when such cells are forced to survive in the absence of contact with a properly formed basement membrane and also when growing as ascites tumor cell clumps in the peritoneal cavity. In support of this possibility, our results show that the enforced down-regulation of Bcl-XL renders the ovarian cancer cells sensitive to anoikis. Adhesion and cell shape normally regulate Bcl-XL expression (12), but Bcl-XL appears to be adhesion independent in transformed (tumorigenic) ovarian epithelial cells, in contrast with normal ovarian epithelial cells.

The importance of Bcl-XL to normal ovarian epithelial cell survival is implicated by the observation that one mechanism for inducing apoptosis in the cells, i.e., the lack of substratum attachment, is associated with down-regulation of Bcl-XL expression. These results suggest that signaling pathways from adhesion receptors converge on Bcl-XL as a key regulator of epithelial cell survival. The cell-substratum adhesion molecules implicated in the induction of apoptosis have yet to be determined. In contrast to normal ovarian epithelial cells, ovarian carcinoma cells plated in three-dimensional culture maintain constant Bcl-XL levels compared with their monolayer counterparts and subsequently survive under such inimical, nonphysiological conditions. The striking in vivo data exist in spite of the fact that Bcl-XL is not completely abrogated. Therefore, it is possible that the Bcl-XL pathway could be a dominant pathway in the induction of anoikis in response to denied substrate attachment.

In conclusion, our work indicates that partial down-regulation of Bcl-XL reverts ovarian carcinoma cells to a state in which cell substratum attachment is necessary for viability. We therefore postulate that targeting anoikis resistance by blocking Bcl-XL-dependent survival pathways may serve as a potentially effective therapeutic strategy for the treatment of such human malignancies as ovarian cancer for two reasons: (a) the ability of the cancer cells to survive spontaneously would be compromised; and (b) targeting Bcl-XL-dependent survival pathways may render the cells more sensitive to chemotherapy-induced cell death. This is because Bcl-XL overexpression has been implicated in the chemoresistance of several types of murine and human cancers (15, 16, 20–22). The same may be true for ovarian cancer (14, 23). Hence, intrinsic or acquired resistance to such drugs as Taxol, cisplatinum, or cyclophosphamide could be delayed, provided relatively selective targeting of tumor cell Bcl-XL expression is feasible, and thus, a good therapeutic index can be achieved. In this regard, i.p. chemotherapy of ovarian cancer (24–26) may make it more feasible to selectively and specifically target Bcl-XL expressed in ovarian cancer cells.

Fig. 4. Suppressed tumorigenicity of HEY cells in vivo upon down-regulation of Bcl-XL. In A, the indicated cell lines were injected s.c. into nude mice, and tumor volumes were measured at the indicated time points. Parental, HEY cells; veccon, vector control cells; and 28, 20, and 18, antisense Bcl-XL clones. The number of mice used in each group was five; bars, SE. This experiment was repeated twice with similar results. In B, the indicated cell lines were injected i.p. into nude mice, and survival times of the mice were determined. The number of mice used in each group was 10. C. Western blot analysis of Bcl-XL in tumors derived from the HEY cells (par), vector control cells (veccon), and antisense Bcl-XL-transfected clones (28, 20, and 18). Tumors were extracted from mice, cell lines were reestablished in tissue culture, and levels of Bcl-XL were analyzed. Each lane represents a tumor derived from an independent mouse. The membrane was reprobed with an anti-Cdk4 antibody as a loading control.
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Induction of Anoikis and Suppression of Human Ovarian Tumor Growth in Vivo by Down-Regulation of Bcl-XL

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