Antisense RNA Down-Regulation of bcl-xL Expression in Prostate Cancer Cells Leads to Diminished Rates of Cellular Proliferation and Resistance to Cytotoxic Chemotherapeutic Agents

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ABSTRACT

bcl-xL is a Mr 26,000 bcl-2 homologue that is highly expressed in prostate cancer cells. In previous studies, the down-regulation of its expression by antisense oligonucleotides led to resistance. In this work, the 445-bp 5’ terminus of the bcl-xL cDNA was cloned in the antisense orientation and stably transfected into DU145 and LNCaP prostate cancer cells. In the DU145 (and to a lesser extent the LNCaP) transfectants, phenotypic changes (versus mock-transfected cells) included an increase in doubling time (from 36 to 175 h) in the clone in which bcl-xL protein expression was 25% of control. The transfectants did not demonstrate characteristic apoptotic changes, as demonstrated by 4’,6-diamidino-2-phenylindole staining, lack of either DNA laddering, caspase-3 activation, or poly(ADP)ribose and lamin cleavage, and the absence of a significant sub-G0 population. Cell cycle analysis demonstrated an increase in a tetraploid population (from 28% to 66%), as well as the appearance of a hypertetraploid population. Levels of cIAP-1 protein were almost undetectable in the mock cells but increased at least 25-fold in the DU145 transfectants. The down-regulation of bcl-xL in both DU145 (and to a much lesser extent in LNCaP) cells led to their resistance to cytotoxic agents, including docetaxel, mitoxantrone, etoposide, vinblastine, and carboplatin. Reversion of bcl-xL expression in stable DU145 transfectants to nearly the levels found in the mock-transfected cells was accomplished by retroviral infection of the cells with a bcl-xL sense cDNA under control of a proliferon promoter. This led to a dramatic increase in the growth rate and in BrdUrd incorporation, as well as a sharp decrease in the expression of cIAP-1 protein. Overall, these findings highlight the adaptability of prostate cancer cells to loss of bcl-xL and suggest that in addition to its prosurvival role, bcl-xL protein may also be involved in the regulation of the rate of cellular proliferation.

INTRODUCTION

The intracellular processes of cell division, differentiation, and death are under strict regulatory control. Disturbance of this balance can result in inappropriate cell accumulation (e.g., as in the neoplastic process), or in excessive cell loss via the process of apoptosis, as occurs, for example, after treatment of cells with cytotoxic chemotherapy. An increasing number of genes have been discovered that are involved in regulation of apoptosis. bcl-xL and bcl-2 are Mr 26,000 proteins that are strongly antiapoptotic members of the bcl-2 family, a group that also includes proapoptotic members, including bax. These molecules seem to be predominately integral membrane proteins and are found in the outer mitochondrial membrane, endoplasmic reticulum, or outer nuclear membrane (1–3). They are capable of forming ion channels in artificial membranes (4) and can control caspase activation (5), mitochondrial membrane depolarization (6–8), generation of reactive oxygen intermediates (9, 10), and transmembrane calcium fluxes (11, 12).

The antiapoptotic members, at least in part by correcting a defect in ATP/ADP exchange across the mitochondrial membrane (13), can block the mitochondrial release of cytochrome c into the cytosol, where it forms a complex with ATP, apaf-1, and procaspase 9. Procaspase 9 is then cleaved to active caspase 9, which cleaves and activates caspase 3. The two activated caspases can then initiate the cleavage of many other substrates (e.g., PARP and lamin) that are characteristic of apoptotic cell death. bcl-xL and bcl-2 may also act as docking factors for cytochrome c and apaf-1, and in doing so may inhibit their proapoptotic function (14–16).

Many experiments have demonstrated that forced overexpression of bcl-2 and bcl-xL in cells can protect them from apoptosis induced by antineoplastic agents (17–23). In the murine IL-3-dependent prolymphocytic line FL5.12, bcl-xL greatly reduced the proapoptotic effects of bleomycin, cisplatin, etoposide, vincristine, and doxorubicin (20). Forced overexpression of bcl-2 (24) or bcl-xL (25, 26) markedly desensitized prostate cancer cells to the cytotoxic effects of several chemotherapeutic agents, both in tissue culture and in vivo as well. However, these demonstrations of chemoresistance have been in the presence of artificially induced high concentrations of bcl-xL or bcl-2, and it is not clear that results obtained from forced overexpression experiments in cells are relevant to cellular conditions during “basal” expression.

The antisense oligonucleotide biotechnology has been used recently to “specifically” down-regulate bcl-xL protein expression (27) in prostate and bladder carcinoma cells (25, 26), where significant resistance was observed. However, this approach to gene knockout has been controversial, because interpretation of data can be problematic. This has been, at least in part, attributable to the presence of nonsequence-specific effects, especially when oligonucleotides with phosphorothioate backbones have been used (28). Furthermore, the duration of antisense down-regulation of a specific target may be transient; in our hands (25, 26), down-regulation of bcl-xL expression by an antisense oligonucleotide could be maintained for only ~3 days before levels recover.

In theory, the antisense RNA biotechnology offers a convenient way to avoid the problems inherent in the use of antisense phosphorothioate oligonucleotides (29, 30) including transience, nonsequence specificity, and the RNase H-induced cleavage of nontargeted mRNAs (31). Thus, to determine whether bcl-xL knockout by antisense DNA and RNA technologies produced similar phenotypes, we created stable transfectants of DU145 and LNCaP human prostate cancer cells that express a truncated antisense bcl-xL mRNA. Several stable clones were isolated in which bcl-xL protein levels were greatly diminished. However, in what we believe is a novel and important finding, this chronic antisense RNA suppression of basal bcl-xL expression yielded transfected cells with very low rates of cell growth. In addition, reinduction of bcl-xL protein expression after retroviral infection with a plasmid containing the sense bcl-xL cDNA caused a reversion of the cell growth rates. Also surpris-
ingly, cells that expressed low levels of bcl-xL protein did not demonstrate an apoptotic phenotype but were actually chemoresistant compared with control cells. This, perhaps in part, may have been attributable to dramatic, compensatory up-regulation of the expression of cIAP-1 and XIAP, two proteins that are capable of binding to and blocking the activation of caspases (32–34).

MATERIALS AND METHODS

Cell Culture, Expression Vector Constructs, and DNA Transfection Assays. Human prostate carcinoma DU145 and LNCaP cells were obtained from American Type Culture Collection (Rockville, MD). To create ASbcl-xL, an antisense bcl-xL expression vector, and pSFFV-neo cell lines, transfection with either pSFFV-ASbcl-xL or pSFFV-neo (35) was performed using Lipofectin (Life Technologies, Inc., Grand Island, NY) as described previously (36). The pSFFV-ASbcl-xL plasmid was created by cloning a 433-bp 5′-terminal fragment of the bcl-xL cDNA in the antisense direction into the neomycin-resistant expression vector pSFFV-neo. Neomycin-resistant cells were selected with 1 mg/ml G418 and were cloned by limiting dilution. Twenty-nine pSFFV-ASbcl-xL clones from both DU145 and LNCaP cell lines were screened for bcl-xL expression. Four clones of DU145 cells (D1, D2, D3, and D4) and three of LNCaP cells (L1, L2, L3, and L4) with different degrees of bcl-xL down-regulation were selected.

Retroviral infection with the pMSCV system (Clontech Laboratories, Palo Alto, CA) was used for bcl-xL reversion in ASbcl-xL clone D4. The selectable marker, neomycin, is under control of the SV40 virus early promoter. Detection of bcl-xL protein expression in LNCaP and DU145 cells stably transfected with pSFFV-neo AS-bcl-xL. Twenty μg of total protein were loaded per lane and analyzed by Western blot using the anti-bcl-xL polyclonal rabbit antibody. Relative amounts of bcl-xL protein were quantitated by laser scanning densitometry.

The presence of the antisense transcripts was verified by an RNase protection assay. The 445-nt protected fragment that hybridized to the antisense mRNA was observed in the D3 and D4 transfected clones and was not observed in either wild-type DU145 or DM control cells. The 310-nt protected fragment, which corresponds to the bcl-xL sense mRNA, was observed in each clone analyzed.

Fig. 1. Down-regulation of the bcl-xL protein expression in the DU145 stably transfected cells. A, plasmid map of the bcl-xL antisense expression vector pSFFV-ASbcl-xL. A 433-bp 5′-terminal fragment of the bcl-xL cDNA was inserted in the antisense orientation into the KpnI/EcoRI sites of the expression vector pSFFV-neo. The selectable marker, neomycin, is under control of the SV40 virus early promoter. B, detection of bcl-xL protein expression in LNCaP and DU145 cells stably transfected with pSFFV-neo AS-bcl-xL. Twenty μg of total protein were loaded per lane and analyzed by Western blot using the anti-bcl-xL polyclonal rabbit antibody. Relative amounts of bcl-xL protein were quantitated by laser scanning densitometry. C, detection of antisense bcl-xL mRNA in the transfected cells by RNase protection assay. The 445-nt protected fragment that hybridized to the antisense mRNA was observed in each clone analyzed.

Fig. 2. Effect of the bcl-xL protein down-regulation on the cellular proliferation rate. Growth curves of the DM control cells and ASbcl-xL D1–D4 stable transfectants are shown. Relative cell viabilities, as determined by MTT assay as described in "Materials and Methods," were plotted on a semilogarithmic scale for each clone and normalized to the day 1 viability of the corresponding clone. Experiments were repeated seven times, and data are expressed as the means; bars, SE. ○, DM clone; ●, D1 clone; ▲, D3 clone; ■, D4 clone.

Fig. 3. Down-regulation of the bcl-xL protein expression in the DU145 stably transfected cells. A, plasmid map of the bcl-xL antisense expression vector pSFFV-ASbcl-xL. A 433-bp 5′-terminal fragment of the bcl-xL cDNA was inserted in the antisense orientation into the KpnI/EcoRI sites of the expression vector pSFFV-neo. The selectable marker, neomycin, is under control of the SV40 virus early promoter. B, detection of bcl-xL protein expression in LNCaP and DU145 cells stably transfected with pSFFV-neo AS-bcl-xL. Twenty μg of total protein were loaded per lane and analyzed by Western blot using the anti-bcl-xL polyclonal rabbit antibody. Relative amounts of bcl-xL protein were quantitated by laser scanning densitometry. C, detection of antisense bcl-xL mRNA in the transfected cells by RNase protection assay. The 445-nt protected fragment that hybridized to the antisense mRNA was observed in each clone analyzed.

The presence of the antisense transcripts was verified by an RNase protection assay. Total cellular RNA was isolated by the use of the TRIzol reagent (Life Technologies, Inc.). RNase protection assays were performed using the HybSpeed RPA kit (Ambion, Austin, TX). For Northern blot analysis of RNA, the total cellular RNA was isolated using the TRIzol reagent. The human cIAP-1 cDNA probe (kindly provided by John Reed, Burnham Research Institute, La Jolla, CA) was 32P-radiolabeled with [α-32P]dCTP by random primer labeling using a commercially available kit (Promega Corp., Madison, WI). The blots were then hybridized with the cDNA probe overnight at 42°C, washed, and exposed to Kodak X-ray film and developed.

Nuclear Staining with DAPI and H&E. DU145 cells (1 × 10⁶ cells/ml) were cultured on polylysine-treated slides in six-well dishes in RPMI 1640 containing 10% FBS + 1 mg/ml G418. Cells were fixed with methanol, washed with PBS, stained with DAPI for 5 min, and observed by fluorescence microscopy. For H&E staining, cells were cultured on glass slides as above.

Chromosome Analysis. Chromosome preparations were produced from logarithmically growing cells via conventional methods as described previously (37).

Western Blotting. This was performed as described previously (38). Cellular protein content was quantitated by the Bradford assay (Bio-Rad, Hercules, CA). A 1:200 dilution of the following antibodies was used: rabbit anti-bcl-xL polyclonal antibody; mouse anti-PARP, anti-lamin, or anti-caspase 3 monoclonal antibodies; goat anti-survivin or anti-caspase 8 polyclonal antibodies were all from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-cIAP-1, anti-cIAP-2, or anti-XIAP polyclonal antibodies were from R&D...
Systems (Minneapolis, MN). After being washed, filters were incubated with a 1:3000 dilution of peroxidase-conjugated corresponding secondary antibody. ECL (Amersham Life Science, Inc, Arlington Heights, IL) was performed according to the manufacturer’s instructions. To detect Bcl-2 protein, a 1:500 dilution of an anti-Bcl-2 mouse monoclonal antibody (Dako, Carpinteria, CA) was added in 1% BSA/TBS. After being washed, filters were incubated for 45 min at room temperature in 1% BSA/TBS buffer [10 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.1% Tween 20] containing a 1:3000 dilution of peroxidase conjugated antimouse secondary antibody. ECL was performed as described above.

Analysis of DNA Content and Distribution. Separate and simultaneous analyses of intracellular DNA content and BrdUrd uptake were performed on a FACScam flow cytometer (Becton Dickinson, San Jose, CA). Cells were fixed with methanol and stained with 0.01 mg/ml of PI. For BrdUrd (Sigma Chemical Co.-Aldrich, St. Louis, MO) incorporation, cells were treated for 24, 48, or 72 h with medium containing 40 μM BrdUrd. The cells were then harvested, fixed with methanol, treated with an anti-BrdUrd antibody (Becton Dickinson, San Jose, CA), and stained with 0.01 mg/ml of PI. PI and BrdUrd were excited at 488 nm. Twenty thousand cells/sample were analyzed at a rate of 100–200 cells/s. Data were analyzed by the CELLQuest software, and the percentage of cells in all phases of the cell cycle was determined as a ratio of the fluorescent area of the appropriate peaks to the total fluorescent area.

MTT Assay for Determination of Cellular Viability. The cytotoxicity of the chemotherapeutic agents was assessed by measurement of cell viability by use of the MTT assay, as described previously (40). The plates were read on a Dynatech MR600 Microplate Reader at 540 nm. All data were normalized relative to the control, nontreated cells of the corresponding clone. Statistical analysis of the results of the MTT experiments was performed using the Analysis ToolPack provided by Microsoft Excel. A Student’s two-sample t test assuming unequal variances was used to determine the equality of the means of two samples. The confidence level (α) was 0.05.

RESULTS

Selection of Stable AS bcl-xL Transfectants. To create the pSFFV-ASbcl-xL plasmid, a 433-bp 5′-terminal fragment of the bcl-xL cDNA was cloned in the antisense direction into the neomycin-resistant expression vector pSFFV-neo (Fig. 1A). (Experiments had also been performed with a full-length antisense cDNA, but no down-regulation of bcl-xL expression was observed.)

Twenty-nine clones derived from the DU145 human prostate cancer cell line that had been transfected with the pSFFV ASbcl-xL vector were screened for levels of bcl-xL protein expression. Four clones (D1, D2, D3, and D4) were selected with different degrees of bcl-xL protein down-regulation, ranging from 75% (clone D4) to 20% (clone D1), as compared with the pSFFV-neo-transfected (DM) DU145 cells (Fig. 1B). In these DM cells, levels of bcl-xL expression were unchanged from the wild-type DU145 cells. Decreased levels of expression of bcl-xL protein have remained stable for >24 months in culture. Three clones, derived from transfected LNCaP cells (L1, L2, and L3), demonstrated different levels of bcl-xL down-regulation, ranging from 30% to almost 100% relative to the LM cells, as measured by Western blotting and laser scanning densitometry.

To confirm that the antisense RNA was expressed, an RNase protection assay was performed in the DU145 transfectants (Fig. 1C). A 445-nt bcl-xL sense transcript and a 310-nt bcl-xL antisense transcript were synthesized, and the ability of these probes to protect RNA generated in vivo was determined. For the 445-nt sense transcript, protection was observed only for the ASbcl-xL clones. As anticipated, the 445-nt protected fragment was not observed in DU145 DM transfectants, which lacked an antisense transcript (Fig. 1C). Alterna-

![Fig. 3. DNA content distribution of the ASbcl-xL-transfected clones. ASbcl-xL transfect DU145 cells (A) and LNCaP cells (B) were stained with 0.01 mg/ml of PI and analyzed by flow cytometry (excitation wavelength, 488 nm). Twenty thousand cells/sample were analyzed at a rate of 100–200 cells/s.](cancerres.aacrjournals.org)
tively, the 310-nt protected fragment, which corresponds to the bcl-xL sense mRNA, was observed in both the wild-type and DU145 DM cells as well as in the transfected clones (D1–4).

Effect of Stable bcl-xL Down-Regulation on Cell Growth. bcl-xL is believed to protect cells from apoptotic cell death induced by a variety of exogenous and endogenous stimuli. If its intracellular presence were eliminated, it might be expected that the rate of spontaneous cellular apoptosis would increase. This might be manifest as a decrease in viability and/or growth rate of a cellular population. Therefore, we examined the effect of the stable down-regulation of bcl-xL on the in vitro growth rate of DU145 and LNCaP prostate carcinoma cells (Fig. 2). The doubling times ($T_D$) of the antisense-transfected cells were calculated from the regression analysis of the growth curves 

$$N = N_0 \exp(kT),$$

where $N$ = cell number, and $k$ is a proliferation rate coefficient that has been determined individually for each exponentially growing cell clone from evaluation by MTT assay of cellular viability (Fig. 2). Doubling times were decreased in the DU145 cells by 5-fold in clone D4 (75% down-regulation of bcl-xL protein expression versus the DM cells; Table 1). In the LNCaP transfectants, the increase in the doubling time versus the LM cells was more modest (~1.5-fold).

Evaluation of Apoptosis. To investigate whether cell growth inhibition was attributable to the induction of cell death (apoptosis), three complementary analyses of DNA fragmentation were performed. These include analysis of DNA fragmentation by either DNA laddering on gel electrophoresis or in vitro determination of cytoplasmic histone-associated DNA fragments (determined by ELISA assay) and analysis of DNA content by PI staining. In both the DU145 and LNCaP cells, lack of induction of apoptosis was confirmed by the virtual lack of DNA fragmentation as indicated by the absence of DNA laddering and by the low levels of histone-associated DNA detected by immunoassay (data not shown). In addition, in both the DU145 and LNCaP transfectants, flow cytometry analysis using PI uptake by fixed cells only demonstrated a very small increase above background in the sub-G0-G1 population compared with the respective LM or DM cells (Fig. 3). Such an increase would have indicated the loss of relatively small DNA fragments resulting from its endonucleolytic cleavage during apoptosis.
Furthermore, even in the DU145 D4 cells, levels of expression of lamin B1, PARP, and both caspase-3 and caspase-9 remained unchanged relative to the DM cells, and no cleavage products of any protein were observed. In addition, no increased caspase-3 cleavage of a synthetic substrate, as determined spectrophotometrically, could be observed in any of the DU145 ASbcl-xL-transfected clones as compared with DM cells (data not shown).

Cell morphology was analyzed by DAPI staining, but none of the typical apoptotic characteristics, such as chromatin condensation or membrane blebbing, were noted. D4 cells, which have maximally down-regulated bcl-xL protein expression (75%), appeared to be larger in size than the DM cells, as seen after staining with both DAPI and H&E (Fig. 4). In addition, some of these cells appeared to be multinucleated, in contrast with what was observed in the DM cells.

Bcl-xL Down-Regulation Is Correlated with Alterations in the Expression of Several Proteins (e.g., XIAP and cIAP-1) That Are Related to the Process of Apoptosis. Substantial alterations in the levels of both pro- and antiapoptotic protein expression were observed in the transfectants. In both the DU145 and LNCaP clones, bcl-xL down-regulation appeared to be associated with a decrease in bcl-2 protein expression. In the DU145 transfectants, perhaps as compensation for this diminution in antiapoptotic activity, levels of expression of the antiapoptotic protein PKC-α increased by ~3-fold. Most strikingly, in the D4 transfectants, the levels of XIAP and cIAP-1 protein expression (which are strongly antiapoptotic) were elevated ~25-fold from the low levels in the DM cells (Fig. 5A). In addition, as determined by Northern analysis, mRNA expression was inversely related to the levels of cIAP-1 protein expression (Fig. 5B). Similar changes but to a lesser extent were observed in the LNCaP transfectants: the increase in cIAP-1 expression in L1 clone was only about 3-fold versus the LM cells. In the DU145 clones, no change in cIAP-2 protein expression was observed, but in the LNCaP clones, cIAP-2 protein levels decreased markedly. In neither cell line were any consistent changes observed in survivin expression versus the pSFFV-neo cells as a function of cell cycle, determined subsequent to nocodazole arrest of cells in G2-M. In addition, as determined by electrophoretic mobility shift assay and monoclonal antibody supershifting, no changes were observed in levels of nuclear NF-κB activity (i.e., binding to its DNA consensus binding sequence) or in nuclear levels of p65 or p50 (not shown). Other apoptosis-related proteins were also altered in the D4 cells. For example, as illustrated in Fig. 6, the expression of bcl-2 declined by 36%, whereas the expression of PKC-α protein increased 2-fold. In contrast, as expected, levels of caspase-3 were not changed.

Antisense RNA Down-Regulation of bcl-xL Protein Expression Is Associated with Resistance of Prostate Cancer Cells to Cytotoxic Chemotherapy. Drug-induced inhibition of the growth of antisense (D4 clone) and DM-transfected cells by several neoplastic agents was demonstrated in vitro. Various concentrations were used of six chemotherapeutic drugs, including paclitaxel, docetaxel, mitoxantrone, etoposide, vinblastine, and carboplatin (Fig. 7 and data not shown). At this point, it is critical to note that values of absorbance derived from the MTT experiments performed on treated cells were all normalized against those obtained from cells grown under identical conditions but not treated with cytotoxic agents. Therefore, the data presented in this work (Fig. 7) do not merely reflect the effect of increased drug concentration on chemosensitivity. Rather, we believe that increased resistance is reflective of a direct or indirect effect of bcl-xL expression.

Significant differences in the IC_{50} between the ASbcl-xL and DM cells were observed. Drug sensitivity decreased with the diminution of
bcl-xL expression, with D4 cells being less sensitive than D1 cells. The LNCaP cells, on the other hand, were not as chemoresistant as the DU145 clones, possibly because of the lack of marked up-regulation of XIAP and cIAP1, both associated with resistance to cytotoxic agents.

**Analysis of DNA Content and Distribution.** Flow cytometric analysis of the DNA content of PI-stained DU145 or LNCaP pSFFV-neo-transfected cells demonstrated the typical distribution of DNA content (G1-G1, 81%; S, 7%; G2-M, 12%). However, in both the LNCaP and DU145 ASbcl-xL transfectants, we observed a dramatic redistribution of intracellular DNA content, which was particularly evident as bcl-xL protein expression declined. The G1-G1, 2N peak decreased strikingly, whereas the 4N peak had increased relative to it, while an 8N peak appeared (Fig. 8, B–E). In the DU145 transfectants, the decrease of bcl-xL protein expression correlated with the change in the DNA content distribution: the lower the bcl-xL expression, the higher the percentage of "8N" cells (Table 1).

Cells incorporated BrdUrd during the replication of DNA, i.e., during S-phase. To determine the relative population of replicating cells, the DU145 transfectants were treated with BrdUrd for either 24, 48, or 72 h. Three-dimensional representations of population distributions were derived via flow cytometry for the BrdUrd and PI staining of DM and DU145 ASbcl-xL transfectants. Two peaks were found upon analysis of the control DM cells. Each was composed of a population that had incorporated BrdUrd: one corresponding to the G1 ("2N" DNA content) and the G2-M ("4N" DNA content) phases of the cell cycle. In contrast, in the DU145 and LNCaP antisense transfectants, two distinct populations for each phase of the cell cycle were observed after BrdUrd uptake.

One represented a population of cells that incorporated BrdUrd [BrdUrd(+)] and another a population of cells that did not do so [BrdUrd(−)]. In the DU145 transfectant D4 cells, ~48% of the cells did not incorporate BrdUrd on the time scale of the experiment (72 h). These data are consistent with the observed slow growth rate of these cells as evaluated by MTT assay.

With decreasing bcl-xL protein expression in the DU145 transfectants, a dramatic increase in the BrdUrd(−) population was observed, as well as an increase in the "polyploid" cell population. For example, clone D4, which had the highest degree of bcl-xL down-regulation (75%), is described by "4N" and "8N" populations (Fig. 8E). The appearance of the "8N" population and disappearance of the "2N" population correlates with the diminution of bcl-xL protein expression and with the decrease of the cellular proliferation rate (Table 1). Thus, in clone D4, 34% of cells were polyploid (doubling time Td, 175 h), compared with only 2.5% in clone D1 (20% bcl-xL down-regulation doubling time Td, 90 h), and to essentially 0% (Td, 36 h) in the DM transfectants.

**Cytogenetic Studies.** To assess the increase in cell ploidy that appeared to be a consequence of the down-regulation of bcl-xL expression, the DU145 DM and DU145 ASbcl-xL transfectants were analyzed for chromosome number. Although the DM cells were near-triploid, modal chromosome numbers in the ASbcl-xL transfectants varied from hypotriploid to hypertetraploid. These data are summarized in Table 2. The decrease in bcl-xL protein levels correlated with the increase in chromosome number in the DU145 transfectants, with the largest number of chromosomes, n = 100 ± 6, observed in the D4 clone.

**Reversal of the Down-Regulation of bcl-xL Expression in the D4 Clone.** To determine which phenotype changes could be attributed to the knockout of bcl-xL in the D4 clone, the cells were transfected with the pMSCV/bcl-xL vector. Two clones (D4α and D4γ) with 3–4-fold increased expression of bcl-xL protein versus the D4 cells (75 and 88% of the expression of the DM control cells) were obtained (Fig. 9). Cellular growth rates of these revertant clones increased while the DNA content distribution remained unchanged, as determined by flow cytometry (Fig. 10). In these revertant clones, the decrease in bcl-xL protein levels correlated with the increase in chromosome number in the DU145 transfectants, with the largest number of chromosomes, n = 100 ± 6, observed in the D4 clone.

**Table 2** Karyotype analysis of the DU145 ASbcl-xL transfectants

<table>
<thead>
<tr>
<th>Clone</th>
<th>Chromosome number</th>
<th>Average n = 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>neo-pSFFV</td>
<td>45–72</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>D1</td>
<td>52–109</td>
<td>70 ± 21</td>
</tr>
<tr>
<td>D2</td>
<td>50–110</td>
<td>67 ± 22</td>
</tr>
<tr>
<td>D3</td>
<td>52–106</td>
<td>88 ± 17</td>
</tr>
<tr>
<td>D4</td>
<td>77–106</td>
<td>100 ± 6</td>
</tr>
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population doubling times decreased dramatically to 55 and 60 h, respectively, compared with 175 h in the original, D4 clone, and 36 h in the DM clone (Fig. 11). These data are in agreement with the analysis of the BrdUrd uptake data in the revertant clones. After 48 h, 100% of both the D4α and D4γ clones were BrdUrd(+), as compared with only ~50% of cells of the D4 clone after 72 h (Fig. 10).

Western blot analysis of IAP family proteins also demonstrated reversion of the expression of cIAP-1 protein in the D4α and D4γ clones from the high levels observed in the D4 clone to the original levels of expression of cIAP-1 protein in the revertant D4 clone, as assessed by MTT assay (Fig. 12).

DISCUSSION

Antisense RNA inhibition of gene expression can occur at multiple molecular levels, including altering transcription of a target gene, facilitating the degradation of double-stranded mRNA and preventing translation of mRNA into protein by inhibiting its binding to the ribosome (38, 41). This strategy has been used previously to downregulate the expression of bcl-2 protein in tissue culture (42–46), but to our knowledge has not been used to down-regulate the expression of bcl-xL.

Recently, evidence for the ability of both bcl-2 and bcl-xL to act as caspase-independent effector proteins has emerged. Current data consistently suggest their involvement in the regulation of rate of progression through the cell cycle (47–49). For example, both bcl-xL and bcl-2 appear, under some conditions, to restrain the reentry of cells in G0 into the S-phase of the cell cycle (50, 51). With respect to the function of bcl-xL, these results appear opposite to those we describe, but it should be noted that the experiments cited were performed in promyelomonocytic FDC-P1 cells and in a context in which levels of wild-type bcl-xL protein were unchanged. In U937 monocytic cells, however, overexpression of bcl-xL was associated with more rapid growth kinetics as compared with the parental cells (52). Our data demonstrating the relatively rapid growth rate of the bcl-xL revertant D4α and D4γ cells are consistent with this observation. Furthermore, our observations allow us to propose that bcl-xL has at least two functions; at relatively high concentration, as when forcibly overexpressed, bcl-xL can partition into mitochondrial membranes and regulate their biological activities. At lower concentrations, which can
only be observed when bcl-xL expression is reduced below a “basal” level, it acts, by a mechanism as yet unclear, as a regulator of the rate of cell growth.

Previous data (49) have suggested that, in the setting of mitotic spindle damage and loss of p53 expression, bcl-xL-protected cells will have relatively high rates of tetraploidization. [Indeed, tetraploidy and aneuploidy are relatively common features of advanced clinical prostate cancer (53, 54).] However, in contrast to this work, both the L1 clone (wild-type p53; 99% bcl-xL down-regulation) and the D4 clone (mutant, nonfunctional p53; 75% bcl-xL down-regulation; Ref. 55), perhaps because of damage to the mitotic spindle, have undergone endoreduplication and have high modal chromosome numbers. In addition, because of the correlation between modal chromosome number and bcl-xL content, at least in clones D1–D4, it is also possible that bcl-xL, via an unknown mechanism, is protecting the mitotic spindle apparatus. However, because of the clonal selection process, it is not possible to absolutely determine whether the endoreduplication is either a cause or consequence of bcl-xL down-regulation. Nevertheless, as demonstrated by flow cytometry and BrdUrd uptake studies, neither the D4 nor the L1 cells appeared to be arrested in any phase of the cell cycle. Rather, they seemed to progress through it at a very slow rate compared with their respective pSFFV-neo control cells, with cell doubling times increasing as much as 5-fold (from 36 h in the DM cells to ~175 h in the D4 clone; Table 1). In contrast, in the revertant clones, the doubling times decreased dramatically to 55 h (D4α clone) and 60 h (D4γ clone), whereas BrdUrd incorporation concomitantly increased. Taken together, these results strongly suggest that bcl-xL expression controls the rate of progression of cells through the cell cycle. Certainly, however, it may not serve this function in other cell types, and this observation may be unique to prostate cancer cells.

In contrast to what we observed with this antisense RNA approach, a chemosensitive phenotype was observed when human prostate (25), bladder (26), or keratinocytes and epithelial cells (56) were treated with antisense bcl-xL oligodeoxynucleotides. However, it should be noted that the antisense DNA down-regulation of bcl-xL expression occurs over relatively short time periods (on the order of 2–3 days), whereas antisense RNA down-regulation of bcl-xL in the D4 and L1 clones has been stable over a multyear period. This stability has undoubtedly enabled some degree of cellular compensatory adaptation to occur because of the chronically low levels of bcl-xL protein expression and may lead to the up-regulation of compensatory, anti-apoptotic proteins such as cIAP-1 and XIAP. The suggestion is strengthened by the fact that in the D4α and D4γ revertant clones, not only was a diminution in cIAP-1 expression levels observed but, in addition, the D4α cells became as sensitive to mitoxantrone as the original DM cells.

cIAP-1, cIAP-2, and XIAP are members of the IAP family, a group of proteins that are highly conserved across species (57). XIAP (32), cIAP-1, and cIAP-2 (33) bind to and directly inhibit caspase-3 and are capable of protecting cells against many proapoptotic signals, including those delivered by cytotoxic chemotherapy (34). Interestingly, the presence of cIAP-1 has been demonstrated immunohistochemically in 43 of 65 (66%) of in situ specimens of human prostate cancer, compared with only 6% in the adjacent benign prostate epithelium. This level climbs to 94% in patients who eventually developed metastatic disease (58). In this setting, levels of XIAP protein were also up-regulated compared with normal prostate epithelium. The expression of cIAP-1 (59), cIAP-2 (60), and XIAP (61, 62) have been reported to be controlled by NF-kB. However, in the D4 and L1 clones, where maximally up-regulated cIAP-1 and XIAP protein expression could be observed, no NF-kB nuclear activation above that seen in the DM and LM cells could be detected. Furthermore, as cIAP-1 protein expression increased, cIAP-1 mRNA expression dramatically decreased. This suggests the possibility that a negative regulatory feedback loop controls the expression of the cIAP-1 mRNA, an event that has been proposed previously (63). Thus, it seems likely that the dramatic increases in cIAP-1 (and perhaps XIAP) protein expression are attributable predominately to changes in protein stability, perhaps reflected by a change in IAP proteosomal degradation subsequent to ubiquitination (63). Nevertheless, after reversion of bcl-xL expression in the D4 clone, the expression of cIAP-1 protein in the D4α and D4γ clones rapidly reverted to levels as low as that observed in the DM cells, suggesting that the expression of bcl-xL and cIAP-1 in these cells is linked. However, it is unclear whether this linkage is direct or indirect.

It is also of interest that both the antisense RNA and DNA strategies lead to down-regulation of bcl-2 protein expression. In the latter case, this was postulated to be attributable to homology between the bcl-xL antisense oligonucleotide and the bcl-2 mRNA, leading to the RNase H cleavage of a nontargeted message. In the former, as determined by DNA Strider software, no such homology exists. Therefore, it is possible that bcl-xL and bcl-2 are also somehow linked, but further studies will be required to completely analyze the relationship.

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