Increased expression of cyclin B1 sensitizes prostate cancer cells to apoptosis induced by chemotherapy

Lourdes A. Gomez,[5] Alicia de las Pozas,[1] Teresita Reiner,[2] Kerry Burnstein,[3,4] and Carlos Perez-Stable[1,2,4]

[1]Geriatric Research, Education, and Clinical Center and Research Service, VA Medical Center; [2]Department of Medicine, [3]Department of Molecular and Cellular Pharmacology, and [4]Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine; and [5]South Florida VA Foundation, Miami, Florida

Abstract

Chemotherapeutic drugs ideally should take advantage of the differences between transformed and normal cells and induce apoptosis only in cancer cells. One such difference may be the overexpression of cyclin B1 protein in cancer cells, which is required for the proper progression through mitosis. Previously, we showed that treatment of human prostate cancer cells with 2-methoxyestradiol (2-ME) or docetaxel results in an accumulation of cyclin B1 protein and an increase in cyclin B1 kinase activity, followed by induction of apoptotic cell death. Inhibition of cyclin B1 kinase lowers apoptosis induced by 2-ME and docetaxel. In this study, we established a positive correlation between cyclin B1 protein and apoptosis induced by chemotherapy in prostate cancer cells. There is minimal cyclin B1 and induction of apoptosis by chemotherapy in nontransformed cells. LNCaP and PC-3 prostate cancer cells stably overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy. LNCaP cells expressing cyclin B1 small interfering RNA to lower cyclin B1 protein or dominant negative cyclin-dependent kinase 1 to inhibit cyclin B1 kinase show a decrease in apoptosis. Increased sensitivity to apoptosis by overexpression of cyclin B1 may be due to lower Bcl-2, higher p53, and decreased neuroendocrine differentiation. We suggest that a cancer-specific mechanism whereby 2-ME and docetaxel may exert anti–prostate cancer activity is the deregulated activation of cyclin B1 kinase, leading to the induction of apoptotic cell death. Our results also suggest that higher levels of cyclin B1 in prostate cancer cells may be a good prognostic marker for chemotherapy. [Mol Cancer Ther 2007;6(5):1534–43]

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Requests for reprints: Carlos Perez-Stable, Veterans Affairs Medical Center, GRECC (11-GRC), 1201 Northwest 16 Street, Miami, FL 33125. Phone: 305-324-4455, ext. 4391; Fax: 305-575-3365. E-mail: cperez@med.miami.edu

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Introduction

One of the features that distinguish cancer cells from normal cells is uncontrolled cell division, likely resulting from the overexpression of cyclins and the abnormal control of cyclin-dependent kinases (CDK; ref. 1). Cyclins are a family of proteins whose levels vary during the cell cycle to activate specific CDKs required for the proper progression through the cell cycle. Cyclin B1, which is essential for cell cycle progression through mitosis, is overexpressed in a variety of cancers compared with normal cells and tissues (2–4). The deregulated expression of cyclin B1 seems to be closely associated with early events in neoplastic transformation (5).

Antibodies to cyclin B1 have been detected in patients with a variety of cancers including prostate cancer and, therefore, cyclin B1 is considered to be a tumor-specific antigen (6, 7). In addition, the overexpression of cyclin B1 has been identified as a prognostic marker for poor patient outcome in some cancers (8, 9). However, in a recent study, patients with follicular lymphoma expressing higher levels of cyclin B1 showed a better outcome after chemotherapy compared with those with lower cyclin B1 (10). In patients with prostate cancer, high cyclin B1 expression correlated with tumor grade and DNA ploidy, but not with disease recurrence (11). However, in another study, the most powerful predictor of time to relapse of prostate cancer was a high ratio of cyclins A and B to the proliferation marker Ki67 (i.e., the higher the ratio, the longer the time to relapse; ref. 3). Gene microarray studies indicate that the overexpression of cyclin B1 mRNA correlates with undifferentiated metastatic prostate cancer with poor prognosis (12–14). Overall, it is not clear in prostate cancer if the overexpression of cyclin B1 plays a role in disease progression and/or resistance to chemotherapy.

Because the overexpression of cyclins and their associated kinases correlates with increased proliferation of cancer cells, small-molecule inhibitors of CDK activity have been identified and are being investigated in multiple clinical trials as potential chemotherapeutic agents (15). However, some reports cast doubts on the importance of CDK2 inhibition in cancer therapy (16). In addition, knockout mice without cyclin D, cyclin E, CDK2, CDK4, or CDK6 have normal fetal development, suggesting that these genes are not strictly required for cell proliferation (17). Therefore, inhibitors of CDK2, CDK4, and CDK6, which primarily function in the G_1-to-S transition of the cell cycle,

may not be appropriate for cancer therapy. In contrast, deletion of the S and G_2-M cyclin A2 or cyclin B1, which are the partners for CDK1, in knockout mice is associated with an embryonic lethal phenotype, suggesting they are required for cell proliferation (18, 19). Therefore, more recent studies have focused on inhibition of cyclin B1 and CDK1 activity in cancer therapy.

Reduction of cyclin B1 protein in the HeLa cervical carcinoma cell line using small interfering RNA (siRNA) results in decreased proliferation and increased sensitivity to apoptosis induced by paclitaxel (20–22). Other studies have shown that the levels of cyclin B1 can mediate γ-radiation–induced apoptosis and increase sensitivity to paclitaxel and that the specific inhibition of cyclin B1-CDK1 is required for induction of apoptosis by chemotherapy (23–26). Overall, these studies suggest that chemotherapeutic drugs that target cyclin B1-CDK1 may be more appropriate. Whether the levels of cyclin B1 have an effect on apoptosis in prostate cancer and increase sensitivity to chemotherapeutic drugs is not clear.

We have recently shown that treatment of human prostate cancer cell lines with the promising chemotherapeutic drugs 2-methoxyestradiol (2-ME) and docetaxel increases cyclin B1 protein and its associated kinase activity followed by induction of apoptosis (27, 28). Small-molecule inhibitors of CDK1 prevent 2-ME– and docetaxel-mediated increase in cyclin B1–dependent kinase activity and block induction of apoptosis. We hypothesize that 2-ME– and docetaxel-mediated activation of cyclin B1–dependent kinase and G_2-M cell cycle arrest is required for induction of apoptosis in prostate cancer cells. The purpose of the present study was to determine whether altering the levels of cyclin B1 in prostate cancer cells has an effect on apoptosis induced by 2-ME and docetaxel. Our results show that cyclin B1 protein is highest in androgen-dependent and androgen-independent LNCaP prostate cancer cells, which are more sensitive to apoptosis induced by 2-ME and docetaxel, compared with androgen-independent DU-145 and PC-3 prostate cancer cells. An elevation of cyclin B1 protein in LNCaP and PC-3 cells increases apoptosis induced by 2-ME and docetaxel, whereas reduction of cyclin B1 or inhibition of CDK1 activity decreases apoptosis. These results suggest that prostate cancer cells that express higher cyclin B1 protein should be more responsive to apoptosis induced by chemotherapy compared with prostate cancer cells expressing lower levels of cyclin B1.

Materials and Methods

Reagents

2-ME was obtained from EntreMed, Inc. and docetaxel was obtained from Aventis Pharmaceuticals. 2-ME and docetaxel were resuspended in DMSO and aliquots stored at −20°C. 4′-6-Diamidino-2-phenylindole (DAPI) was purchased from Calbiochem. Protease inhibitor cocktail tablets were purchased from Roche Applied Sciences. Coomassie blue was purchased from EMD Chemicals, Inc.

Cell Culture

Human prostate carcinoma cell lines LNCaP, DU-145, and PC-3 were obtained from the American Type Culture Collection (29). LN-AI is an androgen-independent subline of LNCaP, which was spontaneously derived in our laboratory (28). These cells express androgen receptor (AR) and prostate-specific antigen, similar to LNCaP. PC-3/AR cells stably expressing AR and PC-3/*Neo* are the negative control cells (30). LNCaP, LN-AI, DU-145, PC-3, PC-3/*Neo*, and PC-3/AR cells were maintained in RPMI 1640 (Invitrogen) with 5% fetal bovine serum (Hyclone), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin (Invitrogen). Unlike androgen-dependent LNCaP, the LN-AI cells are able to grow for long term in RPMI 1640 with 5% charcoal-stripped fetal bovine serum (Hyclone) and are referred to as LN-AI/CSS. Nontransformed human mesenchymal stromal cells (MSC) derived from bone marrow were obtained from G. D'Ippolito (University of Miami, Miami, FL) and cultured in DMEM (low glucose) with 5% fetal bovine serum and antibiotic/antimycotic (31). The normal rat prostate basal epithelial cell line NRP-152, provided by D. Danielpour, was maintained in HEPES-free DMEM/F12 (1:1, v/v) with 5% fetal bovine serum, antibiotic/antimycotic, 20 ng/mL epidermal growth factor, 10 ng/mL cholera toxin, 5 μg/mL insulin, and 0.1 μmol/L dexamethasone (32).

Treatment with 2-ME and Docetaxel

LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 cells were seeded in 6-cm dishes and allowed to attach overnight. The next day, fresh medium containing 2-ME (1, 2, or 5 μmol/L), docetaxel (0.5, 1, or 10 nmol/L), or DMSO (0.1%) control was added and the cells were cultured for varying times (24–72 h). In all the experiments, floating and trypsinized attached cells were pooled for further analysis.

Western Blot Analysis

Preparation of protein lysates and Western blot analysis was done as previously described (28). We used antibodies specific for cyclin B1 (GNS1), CDK1 (17), Bax (N-20), AR (N-20), Mcl-1 (S-19), survivin (FL-142), p53 (DO-1; Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP; C2-10), E-cadherin (clone 36), Bcl-xL (polyclonal; BD Biosciences PharMingen), cleaved PARP (Asp^{214}), cleaved caspase-3 (9661), X-linked inhibitor of apoptosis (XIAP; Cell Signaling Technology), neuron-specific enolase (clone 5E2; Upstate), and synaptophysin (Zymed Laboratories). Antibodies specific for α-tubulin (TU-02, Santa Cruz Biotechnology) or Coomassie blue staining of total protein on membranes were used for protein loading controls. X-ray films were scanned using an Epson Perfection 2450 Photo scanner and the pixel intensity measured using UN-SCAN-IT digitizing software, version 5.1 (Silk Scientific Corp.). The scanned bands from the same blot were normalized to scanned total protein.

DAPI Apoptosis Assay

For the DAPI staining apoptosis assay, cells were resuspended in 0.6-mL 4% paraformaldehyde/PBS for 15 min, washed with PBS, and resuspended in 0.5 mL of DAPI (1 μg/mL)/PBS for 10 min. Cells were washed with

PBS and 10 μL of concentrated cells added on a microscope slide followed by placement of a coverslip. Cells containing densely stained and fragmented chromatin were identified as end-stage apoptotic using a Nikon fluorescence microscope with a DAPI filter. The number of apoptotic cells in at least 200 total cells was determined from at least four random microscope fields. Changes in apoptosis from 2-ME– and docetaxel-treated cells were determined as percentage of apoptotic cells in at least five different samples from three independent experiments. Minimal apoptosis was detected in control treated cells (<0.5%).

Stable Transfection of Cyclin B1, siRNA Cyclin B1, and Dominant Negative CDK1

To overexpress cyclin B1 in prostate cancer cells, we obtained the pCMX/cyclin B1 expression plasmid from Jonathan Pines (Wellcome Trust/Cancer Research UK; ref. 33). LNCaP cells (90% confluent) were cotransfected with pCMX/cyclin B1 and pCMVneo (for drug selection) using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The negative control was transfection with pCMVneo alone. Cells were grown in media with 400 μg/mL G418, colonies selected, and clones that overexpressed cyclin B1 compared with pCMVneo negative control clones were identified by Western blot. In PC-3, we used FuGene 6 (Roche Applied Sciences) for stable transfection. To lower the cyclin B1 levels, LNCaP cells were cotransfected with pKD-cyclin B1-v2 (siRNA expression plasmid specific for cyclin B1; Upstate) and pCMVneo, and clones containing lower levels of cyclin B1 protein compared with negative control transfected cells (pKD-NegCon-v1; Upstate) were identified. Finally, we isolated LN-AI clones overexpressing dominant negative CDK1, obtained from Sander van den Heuvel (Harvard Medical School, Boston, MA; ref. 34). An LN-AI clone containing the pCMVneo plasmid was used as the negative control.

Transient Transfection of Cyclin B1 siRNA

LN-AI cells were seeded in 12-well plates and transfected the next day with 100 nmol/L siRNA SMARTpool specific for cyclin B1 and siCONTROL nontargeting pool (Dharmacon) using Oligofectamine (Invitrogen), following the manufacturer's instructions. After 48 h, cells were harvested and analyzed for expression of cyclin B1 by Western blot as described above. Subsequently, LN-AI cells were transfected with cyclin B1 for 48 h to reduce cyclin B1 protein, reseeded, retransfected in the presence or absence of 5 μmol/L 2-ME for an additional 48 h, and proteins analyzed by Western blot and compared with siCONTROL siRNA–transfected cells.

Statistical Analysis

Statistical differences between drug-treated and control cells were determined by two-tailed Student's t test, with $P < 0.05$ considered significant.

Results

2-ME can inhibit the growth of a variety of cancer cells, including advanced androgen-independent prostate cancer cells, using a number of diverse mechanisms (35–37). The induction of apoptosis is a requirement for chemotherapeutic drugs to be effective against prostate cancer cells (38). To evaluate the effect of cyclin B1 on apoptosis induced by 2-ME, we used various human prostate cancer cell lines (LNCaP, LN-AI, LN-AI/CSS, DU-145, and PC-3). LNCaP are androgen-dependent and LN-AI, LN-AI/CSS, DU-145, and PC-3 are androgen-independent prostate cancer cells. The results were compared with a primary nontransformed human MSC and rat prostate epithelial NRP-152 cell lines.

Cyclin B1 Protein Level Correlates with Induction of Apoptosis by Chemotherapy

To measure the induction of apoptosis by 2-ME, we did a DAPI staining assay in LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 cells treated with 5 μmol/L 2-ME for 72 h (Fig. 1). We selected this dose because of the increased effect on cyclin B1–dependent kinase, which is important in the induction of apoptosis (27). The results indicated that apoptosis induced by 2-ME was greatest (39%) in LN-AI followed by LNCaP cells (26%). Removal of androgens resulted in decreased apoptosis in LN-AI/CSS (6%) compared with LN-AI cells. The levels of apoptosis in DU-145 (10%) and PC-3 (5%) were similar to those in LN-AI/CSS cells. 2-ME induced minimal apoptosis in nontumorigenic MSC (1%) and NRP-152 (3.5%) cells compared with tumorigenic cells. Similar results were obtained with docetaxel and flavopiridol (a pan-CDK inhibitor; ref. 28). These results indicate that 2-ME, docetaxel, and flavopiridol are more effective in inducing apoptosis in LNCaP and

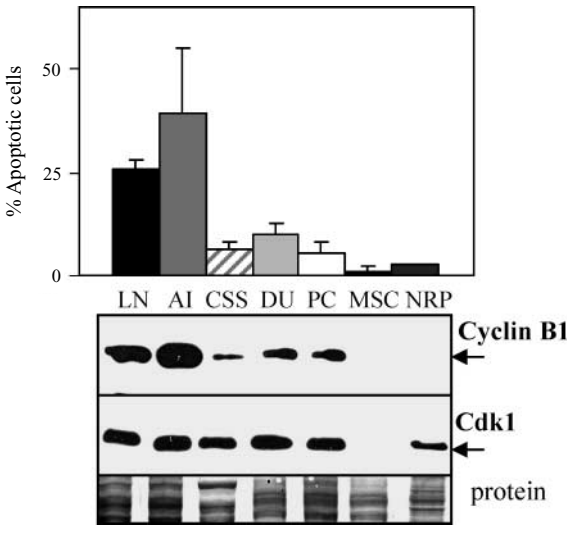

Figure 1. Cyclin B1 protein levels correlate with induction of apoptosis by 2-ME in prostate cancer and nontransformed cells. Top, percentage of apoptotic cells determined by DAPI in LNCaP, LN-AI, LN-AI/CSS, DU-145, and PC-3 prostate cancer cells treated with 5 μmol/L 2-ME for 72 h and compared with treatment of nontransformed MSC and NRP-152 cells. There was minimal apoptosis (<0.5%) detected by DAPI in the 0.1% DMSO control treated cells (not shown). Columns, mean; bars, SD. Bottom, Western blot analysis showing the levels of cyclin B1 and CDK1 in LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 cells (no treatment). Coomassie blue stain of total protein is loading control. Similar results were obtained from three independent experiments.

LN-AI cells as compared with androgen-independent prostate cancer and nontumorigenic cells.

Cyclin B1 protein is overexpressed in cancer cells compared with normal nontumorigenic cells (3, 4, 6). To determine if the levels of cyclin B1 vary between androgen-dependent and androgen-independent prostate cancer cells, we did Western blot analysis of LNCaP, LN-AI, LN-AI/CSS, DU-145, PC-3, MSC, and NRP-152 proteins when cells were 50% to 60% confluent (Fig. 1). The results indicate that cyclin B1 protein levels are greatest in LN-AI followed by LNCaP cells. Removal of androgens results in decreased cyclin B1 protein in LN-AI/CSS cells compared with LN-AI cells and is similar to the levels in DU-145 and PC-3 cells. The levels of cyclin B1 in MSC and NRP-152 cells are very low. In contrast, the levels of CDK1 are similar between the different prostate cancer and NRP-152 cell lines and very low in MSC cells. These results indicate that the levels of cyclin B1 protein correlate with the ability of 2-ME, docetaxel, and flavopiridol to induce apoptosis in prostate cancer cells and in nontransformed cells.

Higher Expression of Cyclin B1 in LNCaP Cells Increases Apoptosis Induced by Chemotherapy

To determine if increased cyclin B1 protein has an effect on apoptosis induced by chemotherapy, we isolated three stably transfected LNCaP clones (LNCaP/B1-15, 23, and 4-1) with increased cyclin B1 protein compared with negative control LNCaP cells (Fig. 2A). To determine if the LNCaP/B1 clones are more sensitive to apoptosis compared with LNCaP/*Neo* cells, we treated cells with low doses of 2-ME (1 and 2 μmol/L). Western blot analysis indicated a significantly greater cleavage of intact PARP to an 85-kDa fragment and an increase in cleaved caspase-3 (measure of apoptosis) in the LN/B1-23 cells compared with the LNCaP/*Neo* cells (Fig. 2B). This result was also confirmed by DAPI analysis (Fig. 2C). Similar results were obtained when LNCaP/B1 clones 15, 23, and 4-1 were treated with low doses of docetaxel (0.5 and 1 nmol/L), flavopiridol (250 nmol/L), doxorubicin (0.25 μmol/L), and camptothecin (10 μmol/L; result not shown). Therefore, these results suggest that higher cyclin B1 protein levels can sensitize LNCaP prostate cancer cells to apoptosis induced by a variety of chemotherapeutic drugs.

Higher Expression of Cyclin B1 in PC-3 Androgen-Independent Prostate Cancer Cells Increases Apoptosis Induced by 2-ME

We investigated in PC-3 cells whether higher expression of cyclin B1 can also sensitize androgen-independent prostate cancer cells to increased apoptosis induced by chemotherapy. PC-3 cells are aggressive undifferentiated androgen-independent prostate cancer cells that are resistant to apoptosis induced by chemotherapy (29). Because most androgen-independent prostate cancer cells in humans express AR, we used PC-3/AR cells that stably express AR (30, 39). Interestingly, PC-3/AR cells express 4- to 5-fold greater cyclin B1 protein compared with PC-3/*Neo* and parental cells (Fig. 3A). PC-3/AR cells undergo greater apoptosis when treated with 5 μmol/L 2-ME, as determined by Western blot (cleaved PARP and caspase-3;

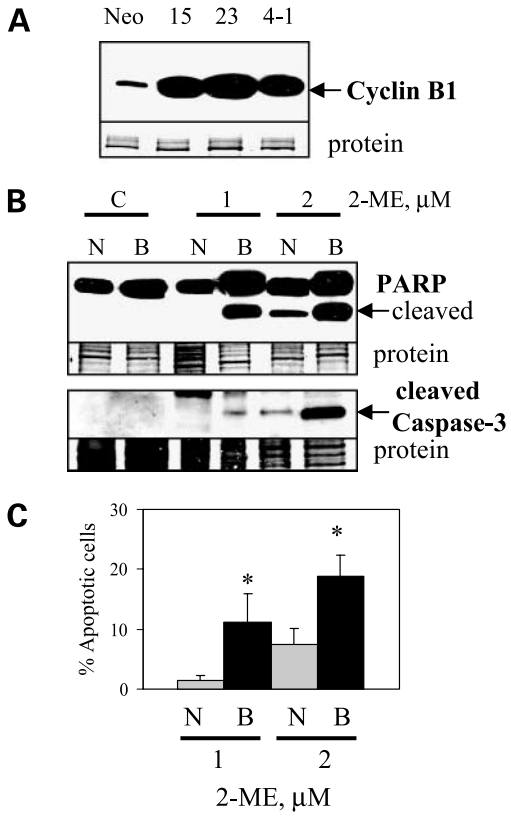

Figure 2. Overexpression of cyclin B1 protein sensitizes LNCaP cells to induction of apoptosis by 2-ME. **A,** three stably transfected LNCaP clones (*15, 23*, and *4-1*) overexpress cyclin B1 more than LNCaP/neo negative control clone (*Neo*), as determined by Western blot. LNCaP/neo cells have similar levels of cyclin B1 protein compared with parental LNCaP cells (not shown). **B,** greater cleavage of PARP and caspase-3 in LN/B1-23 clone (*B*) compared with LNCaP/neo clone (*N*) when treated with 2-ME (1 and 2 μmol/L) for 48 h, as determined by Western blot. No cleaved PARP or caspase-3 was detected in cells treated with vehicle control (*C*). Coomassie blue stain of total protein is loading control. **C,** percentage of apoptotic cells determined by DAPI in LNCaP/neo and LNCaP/B1-23 cells treated with 1 and 2 μmol/L 2-ME for 48 h. LNCaP/B1-23 is more sensitive to apoptosis induced by 2-ME compared with LNCaP/*Neo* cells ($n = 5$, three independent experiments; *, $P < 0.004$, Student's t test). Similar results were obtained with LNCaP/B1-15 and 4-1 clones (not shown). Columns, mean; bars, SD.

Fig. 3B) and DAPI analysis (not shown). To determine if PC-3/AR cells are more sensitive to apoptosis induced by 2-ME because of greater expression of cyclin B1 protein, we isolated stably transfected PC-3 clones expressing higher cyclin B1 protein. The results show that the PC-3/B1-8 clone expresses 2- to 3-fold greater cyclin B1 compared with PC-3/*Neo* cells and undergoes greater apoptosis when treated with 5 μmol/L 2-ME (Fig. 3B). These results suggest that as in androgen-dependent LNCaP cells, higher cyclin B1 protein levels can sensitize androgen-independent PC-3 cells to apoptosis induced by 2-ME.

Reduction of Cyclin B1 Protein with siRNA Decreases Apoptosis Induced by 2-ME

To determine if reduction of cyclin B1 protein has an effect on apoptosis induced by 2-ME, we transiently

transfected siRNA specific for cyclin B1 into LN-AI cells. Cyclin B1 protein was reduced 7-fold compared with LN-AI cells transfected with control siRNA (Fig. 4A). In the presence of cyclin B1 siRNA, there was greater intact PARP in 2-ME–treated LN-AI cells compared with control siRNA (Fig. 4B). There was no effect on PARP cleavage in control treated cells in the presence of cyclin B1 or control siRNA. These results suggest that lowering cyclin B1 protein levels decreases apoptosis induced by 2-ME in LN-AI prostate cancer cells.

To further support a role for cyclin B1 in apoptosis induced by 2-ME and docetaxel, we isolated three stably transfected cyclin B1 siRNA LNCaP clones (LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29) that express 3- to 50-fold less cyclin B1 protein compared with negative control LNCaP cells (Fig. 5A). The results indicated that there was slightly less cleaved PARP and caspase-3 in LNCaP/B1siRNA clones 3, 27, and 29 treated with 5 μmol/L 2-ME compared with negative control LNCaP cells (Fig. 5B). DAPI analysis more clearly showed that there was less apoptosis in LNCaP/B1siRNA clones treated with 2-ME and 10 nmol/L docetaxel compared with negative control LNCaP cells (Fig. 5C). These results provide further evidence that lowering cyclin B1 protein levels in LNCaP cells reduces apoptosis induced by 2-ME and docetaxel.

Dominant Negative CDK1 Reduces Apoptosis Induced by 2-ME

Our previous results suggest that the increase in cyclin B1–dependent kinase activity mediated by 2-ME and docetaxel is required for induction of apoptosis in prostate cancer cells (27, 28). Cyclin B1 associates with CDK1 to form the active kinase complex that is the key initiator of mitosis (2). To further investigate the role of deregulated CDK1 in apoptosis induced by 2-ME, we isolated two stably transfected LN-AI clones overexpressing dominant negative CDK1 (LN-AI/dnCDK-8 and LN-AI/dnCDK-13; Fig. 6A). The results indicated that there was less cleaved PARP and apoptosis in LN-AI/dnCDK1 clones 8 and 13 treated with 2-ME compared with a LN-AI negative control (Fig. 6B and C). These results suggest that inhibition of CDK1 activity lowers apoptosis induced by 2-ME and supports the hypothesis that deregulated cyclin B1-CDK1 activity is important for apoptosis induced by chemotherapy in prostate cancer cells.

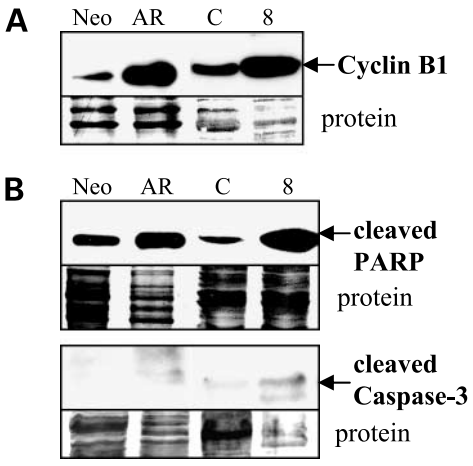

Figure 3. Increased expression of cyclin B1 protein sensitizes PC-3 androgen-independent prostate cancer cells to apoptosis induced by 2-ME. **A,** PC-3/AR and PC-3/B1-8 cells (*AR* and *8*) express greater cyclin B1 protein compared with PC-3/*Neo* cells (*Neo* and *C*), as determined by Western blot. **B,** greater cleaved PARP and caspase-3 in PC-3/AR and PC-3/B1-8 cells compared with PC-3/*Neo* cells when treated with 5 μmol/L 2-ME for 72 h, as determined by Western blot. Coomassie blue stains of total proteins are loading controls. Similar results were obtained from an independent PC-3/B1 clone (not shown).

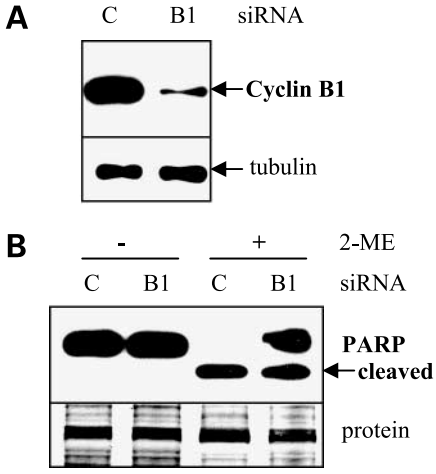

Figure 4. Reduction of cyclin B1 protein by siRNA lowers apoptosis induced by 2-ME in LN-AI cells. **A,** transfection of 100 nmol/L cyclin B1 siRNA (*B1*) for 48 h lowers cyclin B1 protein in LN-AI cells compared with control siRNA (*C*), as determined by Western blot. The same blot was immunostained for α-tubulin showing similar protein loading and specificity of cyclin B1 siRNA. **B,** greater intact PARP in LN-AI cells treated with 5 μmol/L 2-ME and transfected with cyclin B1 siRNA compared with control siRNA, as determined by Western blot. There was no PARP cleavage in cells treated with vehicle (−) and control or cyclin B1 siRNA. Coomassie blue stain of total proteins is loading control.

Overexpression of Cyclin B1 Decreases Bcl-2 and Increases p53 in LNCaP Cells

To investigate why LNCaP cells overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy, we sought to identify differences in the levels of proteins important for apoptosis. Cancer cells resistant to chemotherapeutic drugs often overexpress Bcl-2 and Bcl-xL, which act on the mitochondrial membrane to prevent caspase activation by interfering with cytochrome *c* release (40). In addition, overexpression of inhibitors of apoptosis (IAP) family members like XIAP and survivin blocks apoptosis and increases drug resistance (41). p53, the most commonly mutated gene in human cancers, can also mediate the apoptosis response to chemotherapy (42). The results showed that LNCaP/B1 clones expressed 7- to 15-fold less Bcl-2 protein and 4- to 6-fold more p53 protein compared with LNCaP/*Neo* negative control cells (Fig. 7). There were no changes in the levels of Bcl-xL, Bax, or XIAP.

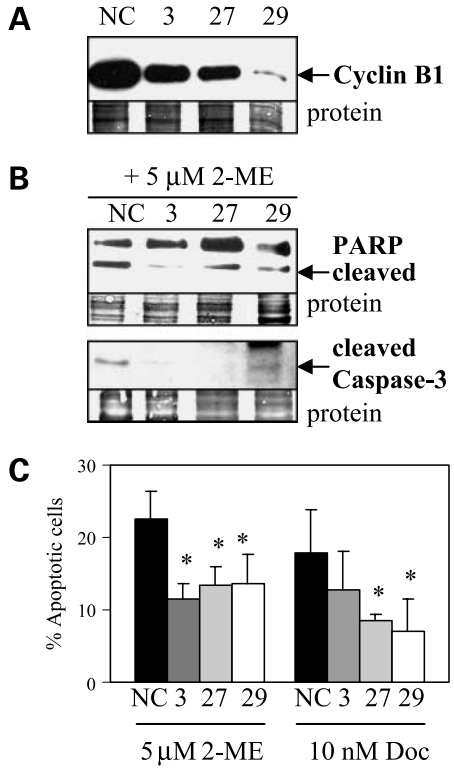

Figure 5. Stable LNCaP cyclin B1 siRNA clones expressing lower cyclin B1 protein are less sensitive to apoptosis induced by 2-ME and docetaxel. **A,** three stably transfected cyclin B1 siRNA LNCaP clones (*3, 27*, and *29*) expressing lower cyclin B1 protein compared with a LNCaP clone expressing negative control (*NC*) siRNA, as determined by Western blot. **B,** slightly less cleavage of PARP and caspase-3 in LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29 compared with negative control LNCaP cells when treated with 5 μmol/L 2-ME for 48 h, as determined by Western blot. Coomassie blue stains of total proteins are loading controls. **C,** percentage of apoptotic cells determined by DAPI in LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29 compared with negative control LNCaP when treated with 5 μmol/L 2-ME and 10 nmol/L docetaxel for 48 h. LNCaP/B1siRNA-3, LNCaP/B1siRNA-27, and LNCaP/B1siRNA-29 cells are less sensitive to apoptosis induced by 2-ME and docetaxel compared with negative control LNCaP cells ($n = 6$, three independent experiments; *, $P < 0.005$, Student's t test). There was minimal apoptosis in vehicle-treated cells (<0.5%; not shown). Columns, mean; bars, SD.

These results suggest that LNCaP cells overexpressing cyclin B1 are more sensitive to apoptosis induced by chemotherapy because they express less Bcl-2 and more p53 proteins.

Overexpression of Cyclin B1 Decreases Neuroendocrine and Epithelial Differentiation in LNCaP Cells

To investigate the effect of overexpressing cyclin B1 on differentiation of prostate cancer cells, we did Western blot analysis of epithelial and neuroendocrine proteins. The normal prostate epithelium consists of secretory luminal, basal, and rare neuroendocrine cells. Intermediate cells coexpressing luminal, basal, and/or neuroendocrine cell proteins have been identified in normal adult prostate and in prostate cancer (43, 44). Studies have shown that hormonal therapy induces neureoendocrine differentiation of prostate cancer, which may contribute to progression into chemotherapy-resistant androgen-independent prostate cancer (45). LNCaP cells are an example of a luminal epithelial/neureoendocrine intermediate prostate cancer cell (46). Our results showed that, compared with LNCaP/*Neo* cells, LNCaP/B1 clones 23, 15, and 4-1 expressed lower levels of neuron-specific enolase and synaptophysin, both being markers of neuroendocrine cells (Fig. 8A). Similar to DU-145 and PC-3 androgen-independent prostate cancer cells, which are more undifferentiated compared with LNCaP cells, there was a decrease in intact and an increase in fragments of the epithelial marker E-cadherin (47) in the LNCaP/B1-23 and LNCaP/B1-15 clones but no changes in the levels of cytokeratins and AR (Fig. 8A and B). E-cadherin fragments have previously been reported in prostate cancer and may be a mechanism to reduce protein levels (48). These results suggest that an overexpression of cyclin B1 decreases neuroendocrine and epithelial differentiation in LNCaP cells.

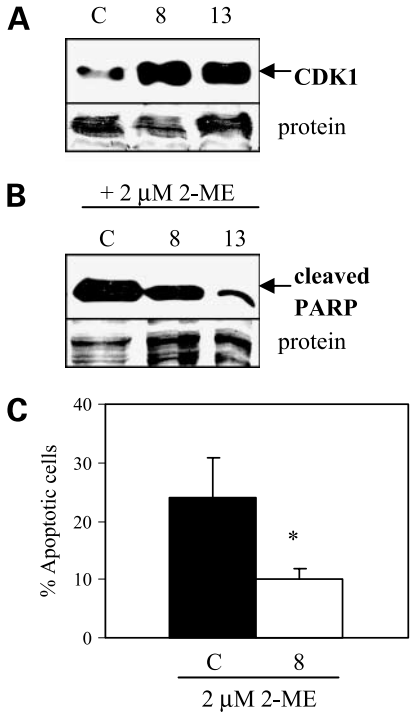

Figure 6. Increased expression of dominant negative CDK1 reduces apoptosis induced by 2-ME in LN-AI cells. **A,** two stably transfected dominant negative CDK1 LN-AI clones (*8* and *13*) overexpress CDK1 compared with LN-AI clone transfected with the negative control pCMV*Neo* plasmid (*C*), as determined by Western blot. **B,** less cleaved PARP in LN-AI/dnCDK1-8 and LN-AI/dnCDK-13 compared with LN-AI/cmv negative control cells when treated with 2 μmol/L 2-ME for 72 h. Coomassie blue stains of total proteins are loading controls. **C,** percentage of apoptotic cells determined by DAPI in LN-AI/dnCDK1-8 compared with LN-AI/cmv negative control cells treated with 2 μmol/L 2-ME for 72 h. Expression of dominant negative CDK1 in LN-AI/dnCDK1-8 cells lowers apoptosis induced by 2-ME compared with LN-AI/cmv cells ($n = 6$, three independent experiments; *, $P < 0.001$, Student's t test). Columns, mean; bars, SD.

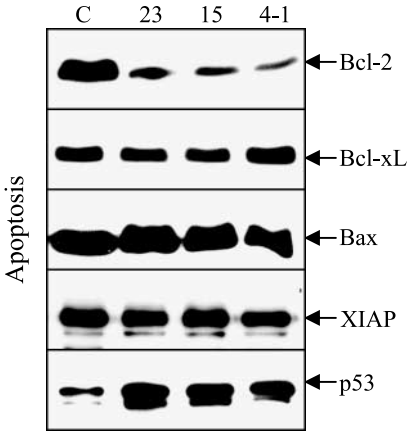

Figure 7. Overexpression of cyclin B1 in LNCaP cells decreases Bcl-2 and increases p53 proteins. The levels of Bcl-2, Bcl-xL, Bax, XIAP, and p53 (proteins important in apoptosis) in LNCaP/B1-23, 15, and 4-1 cyclin B1 – overexpressing clones without drug treatment were determined by Western blot, normalized to Coomassie blue – stained protein (not shown), and compared with LNCaP/*Neo* negative control cells (*C*). There is a decrease in Bcl-2 and an increase in p53 protein in cyclin B1 – overexpressing clones compared with negative control cells. No significant differences were notable in Bcl-xL, Bax, and XIAP proteins.

Discussion

Overexpression of cyclin B1 is associated with transformed cells and is a marker of poor prognosis for a variety of cancers (8, 9). Our results in prostate cancer cells indicate a positive correlation between the levels of cyclin B1 protein and apoptosis induced by chemotherapeutic drugs. The higher the expression of cyclin B1 protein, like in LNCaP and LN-AI prostate cancer cells, the greater the induction of apoptosis by 2-ME, docetaxel, or flavopiridol. In nontransformed human MSC and rat NRP-152 cells, there is minimal expression of cyclin B1 and essentially no induction of apoptosis by 2-ME and docetaxel. LNCaP clones that stably overexpress cyclin B1 protein are more sensitive to induction of apoptosis by chemotherapy, whereas clones that decrease cyclin B1 protein or overexpress dominant negative CDK1 are less sensitive to apoptosis. Among possible mechanisms for increased sensitivity to apoptosis in LNCaP clones overexpressing cyclin B1 may be lower levels of the apoptosis inhibitor Bcl-2 and higher levels of the apoptosis promoter p53. In addition, an overexpression of cyclin B1 in LNCaP cells decreases neuroendocrine differentiation, which is associated with resistance to chemotherapy and poor patient outcome (45). Overall, our results suggest that despite its association with transformed cells, higher levels of cyclin B1 protein in prostate cancer may be a good prognostic marker for chemotherapy.

Immunohistochemical analysis has shown that overexpression of cyclin B1 is a marker for poor prognosis in non–small-cell lung and head and neck carcinomas (8, 9). However, cyclin B1 is not a useful prognostic marker for gastric and colorectal carcinomas (49, 50). In prostate cancer, the results are less clear. In one study, the ratio of cyclins B1 and A to Ki67, considered to be associated with proliferating cells, was correlated with better patient outcome by delaying the recurrence, whereas another study showed that cyclin B1 did not correlate with the outcome (3, 11). Several gene microarray studies, however, suggest that high cyclin B1 mRNA correlates with poor patient outcome (12–14). A recent study in follicular lymphoma shows that high cyclin B1 protein correlates with better response to chemotherapy compared with low cyclin B1 (10). The standard chemotherapy combination for follicular lymphoma includes vincristine, which is a microtubule inhibitor and causes G_2-M cell cycle arrest, similar to 2-ME and docetaxel. Therefore, an increase in expression of cyclin B1 protein may promote greater induction of apoptosis by microtubule inhibitors and result in a better patient outcome. It has also been suggested that rapidly proliferating cancer cells respond better to

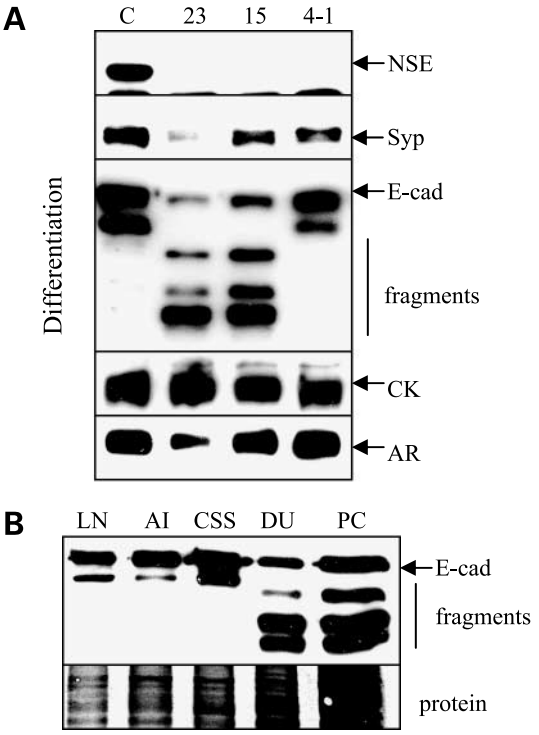

Figure 8. Overexpression of cyclin B1 in LNCaP cells decreases neuroendocrine and epithelial differentiation. **A,** the levels of differentiation markers neuron-specific enolase (*NSE*), synaptophysin (*Syp*), E-cadherin (*E-cad*), cytokeratins (*CK*), and AR in LNCaP/B1-23, 15, and 4-1 cyclin B1 – overexpressing clones without drug treatment were determined by Western blot, normalized to Coomassie blue – stained protein (not shown), and compared with LNCaP/*Neo* negative control cells (*C*). There is a decrease in neuroendocrine neuron-specific enolase and synaptophysin in cyclin B1 – overexpressing clones compared negative control cells. In LNCaP/B1 clones 23 and 15, there is a decrease in 120-kDa E-cadherin (*arrow*) but an increase in E-cadherin fragments (~80, 70, and 65 kDa). No significant differences were notable in cytokeratin and AR proteins when normalized to total proteins. **B,** Western blot showing lower 120-kDa E-cadherin in DU-145 and PC-3 androgen-independent prostate cancer cells compared with LNCaP, LN-AI, and LN-AI/CSS cells. Similar to LNCaP/B1-23 and 15 cells, there is an increase in E-cadherin fragments in DU-145 and PC-3 cells.

apoptosis induced by chemotherapy. However, our results suggest this is not the case in prostate cancer because 2-ME, docetaxel, and flavopiridol induced less apoptosis in the more rapidly proliferating DU-145 and PC-3 cells. In addition, MSC and NRP-152 cells are also rapidly proliferating cells, but 2-ME and docetaxel induce very little apoptosis (Fig. 1). We suggest that 2-ME and docetaxel induce apoptosis in prostate cancer cells and not in normal cells because of increased expression of cyclin B1 protein. Additional trials will be required to determine if a higher expression of cyclin B1 protein correlates with a better response to chemotherapy in prostate cancer.

In the HeLa cervical carcinoma cell line, the lowering of cyclin B1 protein with siRNA results in an increase in apoptosis, even without any drug treatment, suggesting a potential gene therapy strategy (20–22). In contrast, our results showed that lowering cyclin B1 protein in LNCaP or LN-AI prostate cancer cells did not increase apoptosis without drug treatment (Fig. 4). There may be distinct differences between cancer cell types because lowering cyclin B1 protein will signal pathways leading to apoptosis in cervical carcinoma cells, but not in prostate cancer cells. In addition, it is often the case that treatment strategies that are effective in one type of cancer may be ineffective in another type. Therefore, gene therapy strategies that reduce cyclin B1 protein are not likely to be effective for all types of cancer.

The molecular mechanisms of cyclin B1 overexpression in cancer and restricted to minimal levels in proliferating normal cells are not clear. It is likely that one of the mechanisms in cancer cells is by deregulation of the cyclin B1 promoter so that there is a greater initiation of transcription (51). Another possible mechanism in cancer cells is the inhibition of cyclin B1 protein degradation, which is critical for progression through mitosis (2). Our results in prostate cancer showed that when androgens were removed and LN-AI cells converted into LN-AI/CSS cells, cyclin B1 protein levels decreased (Fig. 1). In addition, cyclin B1 protein levels are lower in DU-145 and PC-3 cells, which do not express AR and are not responsive to androgens. Interestingly, in PC-3/AR cells, which stably express AR, cyclin B1 protein levels are 4- to 5-fold higher than in PC-3/*Neo* or parental cells (Fig. 3A). These results suggest that in prostate cancer cells, AR and androgen signaling may increase cyclin B1 protein levels. Whether this is the result of increasing cyclin B1 transcription and/or decreasing protein degradation is currently being investigated in our laboratory. Positive androgen regulation of cyclin B1 mRNA and protein has previously been shown in the CWR22 xenograft model of prostate cancer (52). Other reports have shown that p53 can negatively regulate cyclin B1 promoter and is important in G_2 checkpoint control (53, 54). Our results, however, indicate that overexpression of cyclin B1 in LNCaP cells (wild-type p53) increases p53 protein levels (Fig. 7). In addition, cyclin B1 protein is lower in DU-145 and PC-3 cells, which contain mutations in p53 that render it nonfunctional (29). Therefore, wild-type p53 in prostate cancer cells may increase expression of cyclin B1, although the pathways of how this occurs are not known.

The mechanisms how chemotherapeutic drugs that deregulate (2-ME and docetaxel) or inhibit (flavopiridol) cyclin B1-CDK1 activity can induce apoptosis in cancer cells are poorly understood. Changes in the phosphorylation status of key CDK1 substrates mediated by chemotherapy may be important in the induction of apoptosis. For example, CDK1 phosphorylates the proapoptotic protein BAD to mediate apoptosis in primary neurons (55). However, mutation of the CDK1 phosphorylation site of BAD suggests that it has no role in paclitaxel-mediated apoptosis in nonneuronal cells, including breast cancer (56). CDK1 can also phosphorylate and stabilize survivin, a member of the IAP family (57). The subsequent decrease in cyclin B1-CDK1 activity results in a decrease in the levels of survivin and an increase in sensitivity to induction of apoptosis. However, our previous results suggest that the initial 2-ME– and paclitaxel-mediated increase in cyclin B1–dependent kinase activity is more important than the subsequent decrease in activity for the induction of apoptosis (27). We have preliminary data suggesting that the 2-ME stimulation of cyclin B1 kinase can increase proapoptotic Bak1 and decrease prosurvival AKT in prostate cancer cells (not shown). Further work is required to determine if Bak1 and AKT are important for 2-ME–mediated apoptosis.

What is clear from our results is that the levels of cyclin B1 protein in prostate cancer cells are important for increasing apoptosis by chemotherapy, although the exact downstream mechanisms are not known. One of the proposed mechanisms in LNCaP cells is a decrease in Bcl-2 protein in cyclin B1–overexpressing clones (Fig. 7). This may facilitate the release of mitochondrial proteins to initiate the process of apoptosis through activation of caspase-3 and PARP cleavage (40). Another potential mechanism in LNCaP cells is the increase in wild-type p53 protein in cyclin B1–overexpressing clones (Fig. 7), which can mediate apoptosis induced by 2-ME, docetaxel, and flavopiridol. It is not known whether the overexpression of cyclin B1 has a direct effect on the Bcl-2 and p53 gene promoters and/or protein stability or if it is a consequence of an effect on cell differentiation. Interestingly, an overexpression of cyclin B1 decreases neuroendocrine differentiation in LNCaP cells as shown by lower levels of neuron-specific enolase and synaptophysin (Fig. 8A). Neuroendocrine cells in prostate cancer have been shown to express higher levels of Bcl-2 protein and are known to be more resistant to chemotherapy (58, 59). In addition, a higher neuroendocrine differentiation in patients correlates with poor outcome (45). Therefore, higher levels of cyclin B1 in LNCaP cells promote a cell phenotype that has less neuroendocrine differentiation and is more responsive to chemotherapy. However, a higher level of cyclin B1 also seems to have an effect on lowering epithelial differentiation by decreasing the levels of E-cadherin. This may reflect the association of higher cyclin B1 with a more undifferentiated prostate cancer cell phenotype.

Anticancer chemotherapeutic agents ideally should take advantage of the molecular differences between transformed and normal cells and induce apoptosis only in cancer cells. Our studies suggest that an overexpression of cyclin B1 protein in cancer cells is a reason for the greater induction of apoptosis by 2-ME and docetaxel in malignant but not in normal cells. Our studies also indicate that increased expression of cyclin B1 in prostate cancer cells can mediate a greater induction of apoptosis by chemotherapy. Therefore, it is possible that evaluating the cyclin B1 protein levels in patients with prostate cancer will be an important prognostic marker for use of chemotherapy. However, larger clinical trials will be required to determine if chemotherapy can increase survival in patients with greater cyclin B1 protein.

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