Galectin-3 Regulates Mitochondrial Stability and Antiapoptotic Function in Response to Anticancer Drug in Prostate Cancer

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Abstract

Prostate cancer is one of the malignant tumors which exhibit resistance to anticancer drugs, at least in part due to enhanced antiapoptotic mechanisms. Therefore, the understanding of such mechanisms should improve the design of chemotherapy against prostate cancer. Galectin-3 (Gal-3), a multifunctional oncogenic protein involved in the regulation of tumor proliferation, angiogenesis, and apoptosis has shown antiapoptotic effects in certain cell types. Here, we show that the expression of exogenous Gal-3 in human prostate cancer LNCaP cells, which do not express Gal-3 constitutively, inhibits anticancer drug–induced apoptosis by stabilizing the mitochondria. Thus, Gal-3-negative cells showed 66.31% apoptosis after treatment with 50 μmol/L cis-diammine-dichloroplatinum for 48 hours, whereas two clones of Gal-3-expressing cells show only 2.92% and 1.42% apoptotic cells. Similarly, Gal-3-negative cells showed 43.8% apoptosis after treatment with 300 μmol/L etoposide for 48 hours, whereas only 15.38% and 14.51% of Gal-3-expressing LNCaP cells were apoptotic. The expression of Gal-3 stimulated the phosphorylation of Ser112 of Bcl-2-associated death (Bad) protein and down-regulated Bad expression after treatment with cis-diammine-dichloroplatinum. Gal-3 also inhibited mitochondrial depolarization and damage after translocation from the nucleus to the cytoplasm, resulting in inhibition of cytochrome c release and caspase-3 activation. These findings indicate that Gal-3 inhibits anticancer drug–induced apoptosis through regulation of Bad protein and suppression of the mitochondrial apoptosis pathway. Therefore, targeting Gal-3 could improve the efficacy of anticancer drug chemotherapy in prostate cancer. (Cancer Res 2006; 66(6): 3114-9)

Introduction

Prostate cancer is the most common cancer in men, with ~230,110 new cases and 29,900 deaths annually in the U.S. in 2004 (1). About 10% to 20% of men with prostate cancer present with metastatic disease. Initially, primary androgen ablation therapy leads to a reduction in serum levels of prostate-specific antigen in patients with metastasis of prostate cancer, but in almost every patient, the disease eventually becomes hormone-refractory and apoptosis-resistant (2). The prognosis for these patients is poor because conventional chemotherapeutic drugs have little effect on hormone-refractory prostate cancer and do not provide a marked survival advantage (3). Therefore, it is necessary to clarify the mechanism of chemotherapeutic drug resistance of prostate cancer and develop a novel strategy for the treatment of prostate cancer.

The galectins comprise a family of 14 members of β-galactoside-binding proteins, characterized by their affinity for β-galactosides and by a conserved sequence of the carbohydrate recognition domain that bind to the carbohydrate portion of cell surface glycoproteins or glycolipids. Galectin-3 (Gal-3), a 31-kDa chimeric gene product, is a multifunctional oncogenic protein which regulates cell growth, cell adhesion, cell proliferation, angiogenesis, and apoptosis (4–12). We and others have shown that endogenous Gal-3, which contains the NWGR anti-death motif of the Bcl-2 family, inhibits epithelial cell apoptosis induced by staurosporine, chemotherapeutic agents such as cisplatin, genistein, tumor necrosis factor, and nitric oxide (13–16). We also reported that nuclear export of phosphorylated Gal-3 regulates its antiapoptotic activity in response to chemotherapeutic drugs (17). Phosphorylated wild-type Gal-3 was exported from the nucleus to the cytoplasm and protected cancer cells from drug-induced apoptosis, whereas nonphosphorylated Ser9 mutant Gal-3 was neither exported from the nucleus nor protected cancer cells from drug-induced apoptosis (17). Other investigators also reported that under certain conditions, Gal-3 was found in the cytoplasm and perinuclear mitochondrial membranes (18, 19), where it was involved in the control of apoptosis, possibly through an interaction with the bcl-2 protein (20).

Expression and cellular localization of Gal-3 are important for the prognosis of a variety of cancers. Sanjuan et al. reported on the down-regulation of Gal-3 in colorectal cancer with increased cytoplasmic expression of Gal-3 at more advanced stages (21). Down-regulation of Gal-3 was also observed in prostate cancer, and both nuclear exclusion and cytoplasmic localization of Gal-3 are correlated with cancer progression (19, 22). Furthermore, Califice et al. reported that cytoplasmic Gal-3 expression in LNCaP cells induced tumor growth, invasion, angiogenesis, and decreased inducible apoptosis (23). Here, we investigated the effects of introducing Gal-3 into non-expressing human prostate cancer cells (LNCaP) on their response to proapoptotic chemotherapeutic agents and explored the mechanisms involved in the apoptotic function of Gal-3. We show that the expression of Gal-3 in LNCaP cells inhibits cis-diammine-dichloroplatinum (CDDP)– and etoposide-induced...
apoptosis by regulation of Bcl-2-associated death (Bad) protein, mitochondrial integrity, inhibition of cytochrome c release, and caspase-3 activation.

Materials and Methods

Cell culture. The human prostate cancer cell line LNCaP was from American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in RPMI 1640 containing 2 mmol/L glutamine, penicillin-streptomycin (Life Technologies, Inc., Grand Island, NY) and 10% fetal bovine serum in 5% CO₂ at 37°C using standard cell culture procedure. LNCaP cells were transfected with a Gal-3 expression vector as described previously (24). Briefly, the plasmid pH29.BA2, which uses the human ß-actin promoter to drive expression of the human Gal-3 cDNA, was provided by Dr. Douglas Cooper (University of California, San Francisco, CA). This vector was used for the transfection of subconfluent monolayers of LNCaP cells grown for 1 week prior to transfection in Opti-MEM I medium (Life Technologies) supplemented with 5% FCS. Transfection was done using Lipofectin Reagent (Life Technologies) following the manufacturer's protocol. For each dish, cotransfection was done with 9 µg of pH29.BA2 and 1 µg of pSV2neo containing the neomycin resistance gene. Selection was carried out with G418 (Life Technologies) at a concentration of 300 µg/mL, which was added 48 hours after transfection. Clones of stable transfrectants have been isolated and two of them, 29-11 and 29-23, were used in the present study.

Apoptosis assay. Apoptosis was assessed by measuring propidium iodide permeability (Oncogene, San Diego, CA) using a Becton Dickinson FACScan and CellQuest software. Fragmentation of chromosomal DNA detected as the appearance of hypoploid DNA was measured as previously described (25). Briefly, a total of 1 × 10⁶ parental or Gal-3-expressing LNCaP cells were treated with 50 mmol/L CDDP or 300 mmol/L etoposide for 48 hours at 37°C. After washing, cells were fixed with 80% ethanol for 30 minutes at 4°C, washed with PBS, and treated with RNase A (1 mg/mL in PBS) for 15 minutes at 37°C, followed by staining with propidium iodide (50 µg/mL) for 15 minutes at room temperature.

Caspase-3 activation assay. The active form of caspase-3 was determined by direct staining of cells with a FITC-conjugated rabbit anti-active caspase-3 monoclonal antibody (BD Biosciences, San Diego, CA) followed by FACScan analysis as described previously (25). Briefly, cells were fixed with 1% formaldehyde for 15 minutes at 4°C, washed twice with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes at 4°C and incubated with goat serum. Then, cells were stained with FITC-conjugated anti-active caspase-3 antibody (PharMingen, San Diego, CA). After washing with PBS, cells were again fixed with 1% formaldehyde and analyzed with FACScalibur. To further investigate caspase activity, we measured acetyl-Asp-Glu-Val-Asp-7-aminomethylcoumarin (Ac-DEVD-AMC) activity in control or Gal-3-expressing LNCaP cells treated with 100 mmol/L CDDP as described previously (25). Cells were lysed with cell extraction buffer [20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L DTT] containing 0.03% Nonidet P-40. Lysates were centrifuged at 15,000 × g for 10 minutes, and 50 µL of the cytosolic fraction was incubated for 60 minutes at 37°C in a total volume of 200 µL of caspase buffer [10 mmol/L HEPES (pH 7.5), 50 mmol/L NaCl, and 2.5 mmol/L DTT] containing 25 µmol/L of Ac-DEVD-AMC (Bachem, King of Prussia, PA). -Aminoenamethylene cyanhrome fluorescence, released by caspase activity, was measured at 480 nm using 360 nm excitation wavelength using a Spectra Maxi Gemini fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Caspase activity was normalized per microgram of protein determined by the bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL).

Cytochrome c release. A total of 2 × 10⁶ cells were harvested 24 hours after transfection with 50 mmol/L CDDP. Washed twice with ice-cold PBS, resuspended in ice-cold cell extraction buffer [20 mmol/L HEPES-KOH (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L sodium EDTA, 1 mmol/L sodium EGTA, and 4 mmol/L DTT] containing 250 mmol/L sucrose and protease inhibitor cocktail (Sigma, St. Louis, MO), and incubated for 1 hour at 4°C as described previously (25). The lysates were then passed through a 27 1/2-gauge syringe 10 times and centrifuged at 15,000 × g for 30 minutes at 4°C. The resulting supernatant was analyzed by immunoblot analysis using anti-cytochrome c antibody (Zymed Laboratories, Inc., San Francisco, CA).

Immunofluorescence. Immunofluorescent staining of cells was done as described previously (17). Briefly, the cells were fixed with 4% paraformaldehyde for 5 minutes, permeabilized with 0.5% Triton X-100 for 5 minutes and blocked with 1% bovine serum albumin in PBS for 30 minutes. After the blocking, rabbit polyclonal anti-Gal-3 antibody was added at a 1:50 dilution and incubated for 1 hour. Secondary antibody (FITC goat anti-rabbit IgG; Zymed) was added at 1:200 dilutions and incubated for 1 hour. The stained cells were analyzed by confocal immunofluorescence microscopy using a Zeiss laser scanning microscope 310 (Zeiss, Chester, VA).

Mitochondrial staining. Cells on glass coverslips were incubated with complete medium containing 1.0 µg/mL JC-1 (Molecular Probes, Inc., Eugene, OR) for 30 minutes at 37°C. After washing with PBS at 37°C, cells were placed on a special device, which changes medium from PBS to 100 mmol/L CDDP in PBS. The stained cells were analyzed using confocal immunofluorescence microscopy using a Zeiss laser scanning microscope 310 (Zeiss). The cells were scanned by dual excitation of 488 nm (green) and 568 nm (red) laser lines.

Western blot analysis of cytoplasmic and nuclear protein. Cytoplasmic and nuclear proteins were extracted as described previously (17). Cells were grown to a subconfluent state on 150 mm diameter plates and then exposed to anticancer drugs, CDDP (Sigma) for 48 hours. The cells were then harvested, washed with PBS and resuspended in hypotonic buffer (20 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.1% Triton X-100, 20% glycerol, 5 µg/mL leupeptin, 10 µg/mL aprotinin, and 500 µmol/L phenylmethylsulfonyl fluoride; Sigma). The suspensions were centrifuged at 3,000 rpm for 5 minutes. The supernatant was used as the cytoplasmic fraction. The pellet was resuspended in extraction buffer (20 mmol/L HEPES, 10 mmol/L KCl, 1 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.1% Triton X-100, 20% glycerol, 420 mmol/L NaCl, 5 µg/mL leupeptin, 10 µg/mL aprotinin, and 500 µmol/L phenylmethylsulfonyl fluoride). This suspension was centrifuged at 15,000 × g for 30 minutes. The supernatant contained the nuclear fraction. Protein concentrations were measured using protein assay reagent (Bio-Rad, Hercules, CA). Ten micrograms of cytoplasmic protein and 20 µg of nuclear protein were separated by 12.5% SDS-PAGE by the method of Laemmli and transferred to a polyvinylidene difluoride membrane. This membrane was subjected to immunoblot analysis using rat anti-Gal-3 antibody TIB166 (American Type Culture Collection), anti-Bcl-2 antibody, anti-active Bad antibody (Promega, Madison, WI), anti-phospho-Bad (Ser1³) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome c antibody (Zymed), anti-ß-actin antibody (Sigma), horseradish peroxidase (HRP) anti-rabbit antibody, HRP anti-mouse antibody, and HRP anti-rabbit antibody (Zymed). Western blot analyses were done using the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) as described previously (16, 17, 25). For densitometric analysis of Western blots, Scion Image (Scion, Frederick, MD) was used.

![Figure 1](image)

**Figure 1.** Gal-3 expression in stable transfrectants of LNCaP cells. LNCaP cells and two clones of Gal-3 transfrectants were solubilized and lysates containing 40 µg of protein were subjected to gel electrophoresis and Western blotting. Recombinant Gal-3 (20 ng) served as a positive control. To confirm the equal loading of proteins in each lane, the same membrane was re-probed with anti-ß-actin antibody.
Results

Gal-3 suppresses anticancer drug–induced apoptosis. Two clones of Gal-3-expressing LNCaP cells were isolated. Western blot analysis of Gal-3 transfectants in LNCaP confirmed that the control cells do not express protein, whereas the transfectants express abundant levels of the protein (Fig. 1). To determine whether the expression of Gal-5 in LNCaP cells results in antiapoptotic effects, control and Gal-3-expressing LNCaP cells were treated with 50 μmol/L CDDP or 300 μmol/L etoposide, and cell death was analyzed by flow cytometry after staining with propidium iodide. Following treatment with 50 μmol/L CDDP for 48 hours, control Gal-3-null LNCaP cells underwent 66.3% apoptotic cell death, whereas in the Gal-3-expressing cells, only 2.9% and 1.4% of the cells were apoptotic (Fig. 2A). Similarly, exposure of these cells to 300 μmol/L of etoposide for 48 hours resulted in apoptosis of 43.8% of control LNCaP cells, whereas only 15.3% and 14.5% of Gal-3-expressing LNCaP cells were apoptotic (Fig. 2B). These results indicate that Gal-3 could inhibit apoptosis induced by both CDDP and etoposide.

Gal-3 inhibits cytochrome c release and subsequent caspase-3 activation. Mitochondrial events critical for apoptosis include the disruption of electron transport, loss of mitochondrial transmembrane potential, and the release of cytochrome c (25, 26), resulting in caspase-9 and caspase-3 activation. To examine whether intracellular Gal-3 inhibits the loss of mitochondrial integrity, we analyzed cytochrome c release from the mitochondria. The results show that intracellular Gal-3 inhibits cytochrome c release from the mitochondria (Fig. 3A). Densitometric tracing analysis shows that the level of cytochrome c treated with CDDP in Gal-3-expressing LNCaP decreased by 3.3-fold compared with control cells (Fig. 3B).

Caspase-3 is a critical downstream protease in the apoptotic cascade (27, 28), which is involved in cell death in response to numerous apoptotic stimuli including Fas ligand or tumor necrosis factor-α ligation with its receptor (26, 29). To investigate whether Gal-3-inhibited apoptosis induced by CDDP is the result of inhibition of the caspase pathway, we evaluated the levels of active caspase-3 after treatment with 50 μmol/L CDDP for 48 hours. The results show that Gal-3 inhibited active caspase-3 expression (Fig. 4A). We further measured DEVDase activity in control and Gal-3-expressing LNCaP cells treated with 100 μmol/L CDDP 6 hours after the induction of apoptosis. DEVDase activity in control LNCaP cells treated with CDDP increased by >13-fold above basal levels, whereas its activity in Gal-3-expressing LNCaP cells increased by only 2.5- or 3.0-fold above basal levels (Fig. 4B). These results showed that Gal-3 inhibited cytochrome c release induced by CDDP resulting in inhibition of the caspase pathway. These results suggest that intracellular Gal-3 may directly affect mitochondria integrity leading to the inhibition of the downstream effector caspase-3.
Gal-3 translocation. Gal-3 can shuttle between the nucleus and cytoplasm and can be translocated to the perinuclear membrane following a variety of apoptotic stimuli such as CDDP, serum withdrawal, and staurosporine (17, 18, 30). To determine the effects of CDDP on the localization of the transfected Gal-3 in LNCaP cells, we performed immunofluorescent staining following exposure to CDDP. Before CDDP treatment, Gal-3 was randomly localized in both the nuclear and cytoplasmic compartments (Fig. 5A). However, after 48 hours of exposure to 50 μmol/L of CDDP at 37°C, it was localized exclusively in the cytoplasm (Fig. 5B). The observed change in Gal-3 localization was confirmed by Western blot analysis, which showed that the level of nuclear Gal-3 in LNCaP transfectants decreased after treatment with CDDP (Fig. 5C), whereas the increase of cytoplasmic Gal-3 expression was not obvious, as described previously (Fig. 5D). Western blot analysis of whole cell lysates revealed that exposure to CDDP didn’t change the total levels of Gal-3 (data not shown), indicating that down-regulation in nuclear Gal-3 expression was not due to the degradation of Gal-3, but was due to its nuclear export. To conclude, CDDP exposure induced Gal-3 translocation from the nucleus to the cytoplasm in LNCaP cells.

Gal-3 regulates mitochondrial integrity. We measured and evaluated mitochondrial membrane potential by confocal microscopy after staining living cells with the cationic dye, which exhibits potential-dependent accumulation in mitochondria. Mitochondrial depolarization occurring at the early stages of apoptosis is indicated in JC-1-stained cells by a decrease in the red/green fluorescence intensity ratio. Before exposure to CDDP, the mitochondria in both control and Gal-3-expressing LNCaP cells seemed to be intact. Mitochondrial integrity was evident from the formation of red fluorescence in JC-1 stained cells (Fig. 6A). After exposure to CDDP for 15 minutes, fluorescence of mitochondria in Gal-3-deficient LNCaP cells began to shift from red to green, and by 60 minutes, the mitochondria appeared green in most cells (Fig. 6B and C), indicating depolarization of the mitochondrial membrane. In contrast, mitochondrial membrane potential in Gal-3-expressing LNCaP cells was maintained partially after exposure to CDDP for 60 minutes because many mitochondria seemed to emit red and yellow fluorescence (Fig. 6D-F, 0, 15, and 60 minutes, respectively). These data suggest that intracellular Gal-3 inhibited mitochondrial depolarization and damage induced by anticancer drug.

Regulation of Bad protein by Gal-3. Bad protein is a member of the Bcl-2 protein family that plays an important role in apoptosis. The balance between proapoptotic (e.g., Bad and Bax) and anti-apoptotic (e.g., Bcl-2 and Bcl-XL) members of the Bcl-2 family is critical to control mitochondria-induced apoptosis (31). In our study, Bad protein was expressed in both Gal-3-deficient LNCaP cells and Gal-3-expressing LNCaP cells. The level of Bad was higher in the Gal-3-expressing cells (Fig. 7, compare lanes 3 and 1). Interestingly, treatment with CDDP had an opposite effect on the level of Bad in these cells; it decreased in Gal-3-expressing cells and increased in Gal-3-deficient cells. The level of phospho-Bad increased in Gal-3-expressing cells, whereas phospho-Bad expression was dramatically suppressed in Gal-3-deficient cells (Fig. 7). Bcl-2, Bax, and phospho-MEK expression could not be evaluated because their activities was measured with the fluorogenic substrate Ac-DEVD-AMC, and normalized per microgram of protein. Columns, means of three separate experiments done in duplicate; bars, ±SD.

Discussion

There is no effective treatment for patients with metastasized hormone refractory prostate cancer because this cancer is resistant to anticancer drugs, probably due to enhanced resistance to
apoptosis. We hypothesized that Gal-3, which exhibits antiapoptotic effects in a variety of cells and renders resistance to various proapoptotic agents, may play a role in the drug resistance of prostate cancer. To test this hypothesis, we exploited our finding that LNCaP prostate cancer cells do not express Gal-3 constitutively, as opposed to other prostate cancer cell lines (e.g., DU145 and PC3; ref. 32). By transfecting the LNCaP cells with a Gal-3 expression vector, we were able to compare and contrast the response to proapoptotic agents of isogenic cells without and with Gal-3 protein and thereby assess the potential role and mechanism by which Gal-3 could affect the response to chemotherapeutic agents.

We report here that Gal-3 inhibited apoptosis induced by chemotherapeutic agents such as cisplatin and etoposide in prostate cancer cells, similarly to what we had observed previously in breast cancer cells (13). However, in the present study, we were able to obtain some clues about the mechanism of this effect, as we found that after CDDP treatment, Gal-3 was translocated from the nucleus to the cytoplasm and decreased the level of Bad expression, increased phosphorylation of Bad, and attenuated the depolarization of the mitochondrial membrane. Bad expression and its phosphorylation regulates Bcl-2 expression and the balance between proapoptotic and antiapoptotic proteins is critical to regulate mitochondria-induced apoptosis (30). Based on these data, we propose the following model for the mechanism by which Gal-3 can
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Our data provides the theoretical foundation for a new therapeutic target for improving chemotherapy of prostate cancer. Down-regulation of intracellular Gal-3 by antisense short hairpin RNA approaches may be a useful strategy to enhance the response of prostate cancer to proapoptotic anticancer drugs.

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