Apoptosis Resistance Increases with Metastatic Potential in Cells of the Human LNCaP Prostate Carcinoma Line

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Abstract

The aim of this study was to determine whether stable differences in apoptosis sensitivity were selected for in nonmetastatic and metastatic variants of the LNCaP human prostate carcinoma line that had been isolated from tumors grown orthotopically in the prostate glands and regional lymph nodes of nude mice. The nonmetastatic LNCaP-Pro5 cells were significantly more sensitive to thapsigargin-induced apoptosis than were the metastatic LNCaP-LN3 cells, as measured by viability, DNA fragmentation, and interleukin 1β-converting enzyme family-mediated cleavage of the DNA repair enzyme, poly(ADP-ribose) polymerase. Apoptosis resistance in the metastatic cells was associated with higher levels of expression of the cell death suppressor BCL-2 and lower levels of the death promoters BAX and BAK than were detected in the nonmetastatic LNCaP-Pro5 cells, whereas levels of two other BCL-2 family members (BCL-X<sub>L</sub> and BAD) were indistinguishable. Our data support the hypothesis that apoptosis resistance contributes to prostate cancer metastasis and that elevated expression of BCL-2 is involved.

Introduction

Prostate cancer is the most common malignancy and the second leading cause of cancer-related deaths among men in the United States (1). Despite increased awareness of the disease and improved methods for early detection, a large proportion of patients die of disseminated cancer that is resistant to conventional therapies (2—4). Most of these therapies involve androgen ablation, which is at best palliative and which leads to rapid emergence of androgen-insensitive tumors in the majority of patients by 2—3 years posttherapy (5). Conventional chemotherapeutic agents exhibit little activity against androgen-insensitive tumors (4, 6). Therefore, understanding the biological and molecular mechanisms contributing to prostate cancer progression and drug resistance is crucial to the development of more effective regimens for advanced disease.

A possible breakthrough in our understanding of cancer progression and resistance to therapy has emerged with the hypothesis that both events may involve defects in the regulation of an endogenous “suicide” process known as apoptosis or programmed cell death (7). Apoptosis is an active process of controlled, noninflammatory cellular deletion that complements mitosis in the maintenance of tissue homeostasis (8). There is now abundant evidence that chemotherapeutics and other relevant cancer therapies induce apoptosis in their tumor targets, raising the possibility that disruption of this cell death pathway may contribute to multidrug resistance (7). In theory, suppression of apoptosis may also contribute to tumor metastasis. It has been suggested that the continuous presence of tissue-selective survival signals is required to suppress apoptosis in mammalian cells beyond the blastomere stage of development (9, 10); the requirement for these signals may serve to prevent aberrant cellular expansion and/or migration in normal tissues and allows for the dynamic regulation of tissue size (9). This model would predict that metastatic cells acquire new mechanisms to suppress apoptosis upon leaving the microenvironment of the tissue of tumor origin, either via specific interactions with their new microenvironments, up-regulation of cell autonomous mechanisms, or both.

Recent work has identified a number of oncogenes and tumor suppressor genes that play critical roles in regulating apoptosis. Of particular importance is a family of genes homologous to the human bcl-2 proto-oncogene that plays an evolutionarily conserved role in regulating apoptosis (11, 12). In mammalian cells, this polypeptide family comprises several members, some of which (i.e., BCL-2, BCL-X<sub>L</sub>, and MCL-1) suppress cell death whereas others (i.e., BAX, BCL-X<sub>S</sub>, BAK, and BAD) promote apoptosis. Therefore, differential relative expression of these polypeptides may function in the fine tuning of inherent cell susceptibility to apoptosis (12).

Cumulating evidence supports the involvement of apoptosis in the control of tissue size in the normal prostate and in the effectiveness of current therapies on prostate tumor regression. Using normal rat prostate (13—16), several studies showed that surgical or chemical castration induces a wave of apoptosis, which involves activation of a Ca<sup>2+</sup>-dependent pathway leading to endonuclease activation (17—19). Furthermore, androgen withdrawal can also induce apoptotic cell death in some (but not all) androgen-sensitive prostate tumors grown as xenografts in nude mice or rats (20, 21), suggesting that this mechanism may contribute to the effects observed in patients with early stage disease. Other work indicates that androgens promote BCL-2 expression (22) and that the effects of androgen withdrawal are blocked by enforced overexpression of BCL-2 (23), consistent with some reports of BCL-2 overexpression in androgen-sensitive tumors as detected in immunohistochemical analyses of patient specimens (24—28). However, it has still not been established that prostate tumor progression selects for apoptosis-resistant cells, and it is still not clear that a specific pattern of expression of particular BCL-2 family polypeptides is observed at various stages of tumor progression, in part because of the heterogeneity of patient isolates and differences in disease staging. To address these issues more directly, we exploited a recently developed strategy to isolate nonmetastatic and metastatic variants of a well-characterized human prostate carcinoma line (LNCaP) via orthotopic implantation into the prostate glands of nude mice and subsequent in vivo selection for cells with either low or high metastatic potential. Our characterization of these...
Materials and Methods

Cells, Reagents, and Antibodies. The LNCaP-FGC (LNCaP) human prostate cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). This line is androgen sensitive but possesses a mutation in the hormone-binding domain of its androgen receptor (29). Nonmetastatic (LNCaP-Pro5) and metastatic (LNCaP-LN3) variants were isolated by intraprostatic injection of LNCaP cells and sequential selection for nonmetastatic and metastatic variants as described in detail previously (30). Both lines retain androgen sensitivity in vitro and in vivo, although the LNCaP-Pro5 cells are approximately 2-fold more sensitive to androgen depletion in vitro than in vivo (29). Orthotopic growth led to lymph node metastases in 2 of 17 mice given injections of LNCaP-Pro5 cells and in 13 of 19 mice given injections of LNCaP-LN3 cells (30). Monolayer cultures were maintained on plastic in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS, sodium pyruvate, nonessential amino acids, L-glutamine, vitamins, and antibiotics. The human origin and clonogenic interrelatedness of the lines was verified by cytogenetic analysis (30). Thapsigargin was purchased from Calbiochem (San Diego, CA). Antibodies to human BCL-2 (clone 6C8) and BCL-X (a polyclonal antiserum) were generously provided by Drs. Timothy J. McDonnell and Craig B. Thompson, respectively. A polyclonal antibody to BAX was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and a mouse monoclonal antibody to BAK was from PharMingen (San Diego, CA). A human anti-PARP antiserum was provided by Dr. Antony Rosen (Johns Hopkins University, Baltimore, MD).

Viability Analysis. The cells were harvested in their exponential growth phase by a 2-mm treatment with 0.25% trypsin/0.02% EDTA (w/v). (When this procedure was followed, the viability of harvested cells always exceeded 95%) Cells were seeded into 96-well microculture plates at 5000 cells/well and allowed to attach for 12 h. The medium was removed and replaced with normal medium with or without increasing concentrations of thapsigargin or Adriamycin (doxorubicin), and cells were incubated for 48 h. The medium was then removed and replaced with fresh medium without the cytotoxic agents, and cells were incubated for an additional 24 h. Cell survival/cytostasis was then quantified using the tetrazolium dye MTT as described previously (31). Each experimental data point represented average values obtained from eight replicates, and each experiment was performed in triplicate. Viability measurements were confirmed by trypan blue exclusion.

DNA Fragmentation Analysis. Quantification of DNA fragmentation was accomplished using the diphenylamine reagent as described previously (32). Briefly, cells were harvested by centrifugation, lysed in 0.5 ml of a buffer containing 0.5% Triton X-100, 25 mM Tris (pH 8.0), 10 mM EGTA, and 10 mM EDTA for 15 min on ice, and samples were centrifuged for 20 min at 12,000 × g to separate DNA fragments (supernatants) from intact chromatin (pellets). The DNA content in each fraction was determined using the diphenylamine reagent (33); results are expressed as the percentage of total DNA in each sample that resisted sedimentation at 12,000 × g. Alternatively, for qualitative analysis of oligonucleosomal DNA fragmentation (DNA ladder), the DNA present in the centrifuged supernatants was precipitated overnight at −20°C following addition of two volumes of isopropanol and NaCl (to 0.5 M final concentration). After centrifugation at 12,000 × g for 10 min, precipitates were harvested, dried, and incubated for 1 h in TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA] containing 0.2 mg/ml proteinase K and 1 mg/ml RNase A. The DNA fragments were resolved by electrophoresis for 1 h at 75 V on 1.5% agarose gels preimpregnated with ethidium bromide, detected by UV transillumination, and photographed.

Immunoblotting. Cells were collected at approximately 75—80% confluence with 0.5 mM EDTA in PBS and counted in triplicate. The cells were then lysed as described previously (34). Approximately 20 μg of total cellular protein from each sample were subjected to SDS-PAGE, proteins were transferred to nitrocellulose membranes, and the membranes were blocked with 3% nonfat milk in a Tris-buff ered saline solution containing 0.1% Tween 20 for 2 h at 4°C. The blots were then probed overnight with relevant antibodies, washed, and probed with species-specific secondary (and, in the cases of PARP and BCL-2, tertiary) antibodies coupled to horseradish peroxidase. Immunoreactive material was detected by enhanced chemiluminescence (Amersham Inc., Arlington Heights, IL). Relative polypeptide expression was then quantified with a densitometer (Molecular Dynamics, Sunnyvale, CA). The protein expression experiments were performed in triplicate for each of the proteins described.

Results

We recently isolated nonmetastatic and metastatic variants of the androgen-sensitive LNCaP human prostate carcinoma, termed LNCaP-Pro5 and LNCaP-LN3, respectively, following orthotopic injection and growth in nude mice. To investigate their relative sensitivities to apoptosis, we determined the relative cytotoxicities of thapsigargin and Adriamycin (doxorubicin) on the cell lines by MTT analysis. We chose these compounds because our recent work demonstrated that both are potent inducers of apoptosis in the Dunning rat prostate carcinoma line (35). These compounds act via distinct biochemical mechanisms. Thapsigargin is a diterpene plant product that selectively mobilizes the endoplasmic reticular Ca2+ store, whereas Adriamycin is a cancer chemotherapeutic agent that most likely acts via induction
of DNA damage. Dose-dependent killing was observed in all of the lines in response to both agents; however, the metastatic LN3 subclone was less sensitive to thapsigargin and Adriamycin than were either the parental LNCaP cells or the nonmetastatic Pro5 cells (Fig. 1). Furthermore, the kinetics of apoptosis induced in the LN3 cells by a high dose of thapsigargin (200 nM) were slower than those observed in the parental LNCaP cells or the nonmetastatic Pro5 cells, as measured by qualitative (Fig. 2A) and quantitative (Fig. 2B) DNA fragmentation assays.

Recent work has demonstrated that proteolytic cleavage of PARP by one or more members of the ICE/ced-3 family of cysteine proteases at an internal DEVD (Asp-Glu-Val-Asp) motif leads to the formation of a characteristic 85-kDa fragment that is an early and characteristic feature of apoptosis in diverse cellular systems. Activation of the ICE proteases most likely occurs within the cytoplasm at a point in the apoptotic pathway upstream of the nuclear alterations of apoptosis, including DNA fragmentation. Therefore, to further characterize the block in the apoptotic pathway in the LN3 cells, we measured the kinetics of PARP cleavage as positive evidence for activation of the ICE family following treatment with high-dose thapsigargin. Consistent with the other observations, high-dose thapsigargin induced PARP cleavage more rapidly in the Pro5 cells than it did in the LN3 cells (Fig. 3). The appearance of p85 was blocked by a selective, cell-permeant peptide antagonist of ICE (Z-VADfmk; data not shown), confirming that it was mediated by ICE family protease activation. Thus, ICE family protease activation is delayed in the metastatic LN3 cells.

Several studies using primary patient material have reported that the expression of one or more members of the bcl-2 gene family is altered in progressive prostate cancer. In particular, some investigators have reported that the levels of BCL-2 increase significantly with disease progression, an effect that correlates with the development of

Fig. 2. Time-dependent DNA fragmentation in LNCaP subclones exposed to thapsigargin. A, qualitative analysis. Cells were incubated in the absence or presence of 200 nM thapsigargin for the indicated time periods, and DNA fragmentation was evaluated with agarose gel electrophoresis. The results of one experiment are typical of three independent replicates. B, quantitative analysis. Cells were incubated as described above, and DNA fragmentation was measured using the diphenylamine reagent. Data are the means (n = 3). The differences in the DNA fragmentation levels observed in the LNCaP (□) or Pro5 (■) cells and the LN3 (△) cells were statistically significant (P < 0.01) at 24 h. Bars, SD.

Fig. 3. Time-dependent cleavage of PARP in LNCaP subclones exposed to thapsigargin. Cells were incubated in the absence or presence of 200 nM thapsigargin for the indicated times, and PARP integrity was monitored by immunoblotting. The results of one experiment are typical of three independent replicates.
androgen independence (i.e., resistance to androgen ablation therapy). However, the relative importance of BCL-2 versus other members of the family has been challenged, and the possible relationship between BCL-2 and metastasis has not been determined. Moreover, whether overexpression is achieved solely via the action of the tumor microenvironment or is accomplished via cell-autonomous mechanisms is also not clear. To address these issues, we analyzed the expression of five members of the BCL-2 family of polypeptides in the LNCaP series before and after treatment with thapsigargin. Expression of BCL-2 was significantly higher in untreated LN3 cells than in either the parental LNCaP cells or the nonmetastatic ProS cells (Fig. 4, A and C). Furthermore, the levels of BCL-2 increased in all lines in response to thapsigargin treatment (Fig. 4D), consistent with the observation that steady-state bcl-2 mRNA levels increase following induction of apoptosis by androgen withdrawal in the prostate. Conversely, steady-state levels of the pro-apoptotic BAX (Fig. 4, A and C) and BAK (Fig. 4, B and C) proteins were significantly lower in the LN3 cells than in either the ProS or LNCaP parental cells, although these levels did not change with thapsigargin treatment. In contrast, expression of the cell death inhibitor BCL-XL was virtually identical in all of the lines and was not altered by thapsigargin treatment (Fig. 4, A and C). Similarly, expression of the pro-apoptotic BAD protein was similar in all lines before and after thapsigargin treatment (Fig. 4, B and C). Together, these observations suggest that altered expression of BCL-2, BAX, and BAK may contribute to apoptosis resistance in the metastatic LN3 cells. Furthermore, because these properties are readily observed in vitro, our data argue that a significant portion of their apoptosis resistance is due to intrinsic mechanisms that may be selected for by the tumor microenvironment as a result of growth within the prostate or at metastatic sites in vivo.

Discussion

The purpose of this study was to determine whether nonmetastatic and metastatic variants of the well-characterized LNCaP human prostate carcinoma line exhibited stable differences in apoptosis sensitivity in vitro. Our work was prompted in part by a hypothesis recently advanced by Raff (9), which proposed that cells depend on signals generated by their specific microenvironment to suppress death by default (apoptosis). This model would have important ramifications...
for tumor metastasis, where cells leave their normal microenvironment (and presumably the survival signals found there) behind. The crucial role of the microenvironment in determining tumor metastasis was appreciated more than a century ago by Paget (36) and has been confirmed by extensive work over the past two decades (37). To investigate the possible impact of alterations in tumor microenvironment on apoptosis resistance, we took advantage of a panel of LNCaP subclones that were isolated following implantation and growth in the prostate and regional lymph nodes of nude mice (30). This strategy led to much greater tumorigenicity and metastasis than occurs following ectopic implantation at other sites (38) and allowed for the isolation of subclones with stably different metastatic profiles after several passages in vivo. The LNCaP-Pro5 cells form larger primary tumors than do the LNCaP parental cells but rarely metastasize, whereas the LNCaP-LN3 cells metastasize to the lymph node with high frequency. Here, we show that the nonmetastatic cells are also more sensitive to induction of apoptosis in vitro, indicating that the metastatic cells acquired some intrinsic survival advantage. Coupled with the likely contribution of survival signals emanating from the new microenvironment at the metastatic site, this survival advantage may contribute significantly to their metastatic phenotype.

Emerging evidence indicates that members of the bcl-2/ced-9 family regulate programmed cell death in an evolutionarily conserved manner (11, 12). With respect to prostate cancer, data have been advanced that up-regulation of BCL-2 is associated with cancer progression and the acquisition of an androgen-insensitive phenotype (24), although the relative significance of BCL-2 in the process has more recently been disputed (27). In this study, of the five members of the BCL-2 gene family analyzed, up-regulation of BCL-2 was the most dramatic alteration of expression observed in the metastatic LN3 cells, indicating that it was directly selected for by the metastatic cells in this model. Down-regulation of BAX and BAK was also observed, which may contribute to the apoptosis-resistant phenotype of the cells. Of importance, expression of BCL-XL and BAD were indistinguishable among the cell lines, demonstrating that there was some selectivity in the process. Nonetheless, it is possible that these proteins also play an important role in progression: it is possible that their expression could be altered by signals from the in vivo microenvironment or that they could play important roles at a different step in the process. Consistent with this notion, recent work on human tissue samples suggests that BCL-XL is up-regulated in advanced prostate cancers and tumor metastases (27).

Recent studies indicate that cancer chemotherapeutic agents, radiation, and other clinically relevant treatment regimens can induce apoptosis in their tumor targets (7). Therefore, if tumor progression and metastasis select for apoptosis resistance and alterations in the BCL-2 family expression, it is conceivable that apoptosis resistance is an important cause of drug resistance. Androgen ablation is the current frontline therapy for advanced prostate cancer, and it is possible that the emergence of androgen-insensitive cells that do not respond to such treatment involves the acquisition of intrinsic apoptosis resistance. Consistent with this notion, the LNCaP-Pro5 cells retain high sensitivity to androgen, whereas the LN3 cells are partially androgen resistant (30). The association of this phenotype with elevated BCL-2 expression in vitro and in vivo has important consequences for the design of future therapies, because overexpression of BCL-2 can interfere with diverse triggers for apoptosis, including chemical chemotherapeutic agents and radiation. Thus, strategies aimed at reversing the mal-regulated expression and/or function of BCL-2 and other members of the family in prostate cancer and other solid tumors may hold the greatest promise for reversing drug resistance in the cells. Our ongoing efforts are aimed at more directly defining the roles of apoptosis resistance and BCL-2 family expression in prostate cancer metastasis and chemoresistance in our xenograft tumor model.

References


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