Extended Survivability of Prostate Cancer Cells in the Absence of Trophic Factors:
Increased Proliferation, Evasion of Apoptosis, and the Role of Apoptosis Proteins

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ABSTRACT

This project was undertaken to study the survival properties of various prostate cells, including normal (NHP), BPH (benign prostate hyperplasia), primary carcinoma (PCA), and metastatic prostate cancer cells (LNCaP, PC3, and Du145), in the absence of trophic factors. Cell proliferation and cell death were quantitated by enumerating the number of live cells using MTS/MTS kit and of dead (apoptotic) cells using 4',6-diamidino-2-phenylindole dihydrochloride nuclear staining. These cells demonstrated an overall survivability in the order of BPH < NHP < LNCaP < PC3 < PCA < Du145. Upon growth factor deprivation, NHP/BPH cells rapidly underwent apoptosis, leading to a decreased number of live cells. PCA/PC3/Du145 cells, in contrast, demonstrated an initial phase of aggressive growth during which apoptosis rarely occurred, followed by a "plateau" phase in which cell loss by apoptosis was compensated by cell proliferation, followed by a second phase in which apoptosis exceeded the cell proliferation. LNCaP cells demonstrated survival characteristics between those of NHP/BPH and PCA/PC3/Du145 cells. We concluded that the increased survivability in prostate cancer cells results from enhanced cell proliferation as well as decreased apoptosis. The molecular mechanisms for evasion of apoptosis in prostate cancer cells were subsequently investigated. Quantitative Western blotting was used to examine the protein expression of P53 and P21WAF-1, Bcl-2 and Bcl-X, (anti-apoptotic proteins), and Bax, Bak, and Bad (proapoptotic proteins). The results revealed that, upon trophic factor withdrawal, NHP and BPH cells up-regulated wild-type P53 and proapoptotic proteins Bax/Bad/Bak and down-regulated the expression of P21. Furthermore, NHP and BPH cells endogenously expressed little or no Bcl-2. In sharp contrast, prostate cancer cells expressed nonfunctional P53 and various amounts of Bcl-2 proteins. Upon deprivation, these cancer cells up-regulated P21 and Bcl-2 and/or Bcl-X, response to withdrawal-induced up-regulation of Bax/Bad/Bak or decreased or even completely lost Bax expression and expressed some novel proteins such as P25 and P54/55 complex. These data together suggest that prostate cancer cells may use multiple molecular mechanisms to evade apoptosis, which, together with increased proliferation, contribute to extended survivability of prostate cancer cells in the absence of trophic factors.

INTRODUCTION

Tissue and organ homeostasis is controlled by balanced cell proliferation, cell survival, and cell death. Cell proliferation is driven and regulated by various peptide growth factors and/or cytokines. Cell survival represents the cell's intrinsic life expectancy in the absence of survival factors, many of which overlap with the growth factors/cytokines that drive cell proliferation. Physiological cell death occurs mostly via programmed cell death or apoptosis (1). Apoptosis is a genetically controlled suicidal program that takes place, in many cases, when the cells run out of their survivability in the absence of growth/survival (or trophic) factors, although it can also be triggered by a wide variety of physiological or obnoxious stimuli. It is generally accepted that apoptosis is a dominant feature unless suppressed or overridden by growth/survival factors; therefore, most eukaryotic cells (with the exception of, probably, blastomeres) will undergo apoptosis when deprived of appropriate survival factors (1, 2). Increased cell proliferation, extended cell survival, and diminished apoptosis have all been implicated in tumorigenesis.

There has been a close association between apoptosis and prostate cancer initiation, progression, metastasis, and response to treatment. The growth and survival of normal prostate epithelial cells depend on androgens; therefore, castration or chemical blocking of androgen function leads to prostate atrophy. Prostate cancer is composed of androgen-dependent and androgen-independent cells. Androgen ablation eliminates most androgen-dependent cancer cells by inducing apoptosis but can rarely cure the patients due to the presence of androgen-independent cells and emergence of apoptosis-resistant clones (3, 4). Bcl-2 oncoprotein expression has been correlated with the progression and metastasis of prostate cancer cells and the emergence of androgen-independent cells, as well as the generation of apoptosis- and therapy-resistant cancer cell clones (5–11). On the other hand, androgen-independent prostate cancer cells still retain appropriate apoptotic machinery and can be induced to undergo apoptosis by a wide spectrum of biological and pharmacological treatments, including wild-type P53 and P21 (12–14), prostate apoptosis response-4 (15), prevention of adhesion (16), transforming growth factor β (17), tumor necrosis factor α (18), Fas (19), chemotherapeutic drugs such as cisplatin, camptothecin, teniposide, and vincristine (20, 21), novel chemical entities such as Linomide (quinoline-3-carboxamide; Ref. 22), β-lapachone (23, 24), phenylbutyrate (25), ribonucleotide inhibitors (26), diethylstilbestrol (27), β-l-(dioxolane-cytidine (28), and retinoid derivatives (29), and signal transduction modifiers such as phorbol esters (30–32) and thapsigargin (33). We recently reported apoptosis induction by prostate secretory protein 94 (34) and hydroxamic acid compounds (35, 36) in hormone-refractory human prostate cancer cells.

Little information exists in regard to differential properties of normal and cancerous prostate epithelial cells in terms of sensitivity to apoptosis induction. Furthermore, no systematic studies have been performed to investigate the interrelationship among the proliferation, survival, and apoptosis of various types of prostate cells. Recently, a series of normal (NHP), BPH, and PCA prostate cultures have been established and characterized (37). In this study, we took advantage of these primary cultures and compared their growth and survival properties in the absence of trophic factors with those of the established malignant prostate cancer cell lines, i.e., LNCaP, PC3, and Du145. We further investigated the changes and the potential roles of several apoptosis proteins in these six different cell types upon growth factor withdrawal. The results show that prostate carcinoma cells demonstrate a much higher proliferative potential and survive much longer than normal and BPH cells.
MATERIALS AND METHODS

Cell Culture, Antibodies, and Reagents. PC3, Du145, and LNCaP (FGC-10) cells were obtained from American Type Culture Collection and routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. NHP, BPH, and PCA cells were surgically isolated from clinically diagnosed and pathologically confirmed patients and characterized as detailed previously (37). All of these primary cells were cultured in KBM basic medium (Clonetics) supplemented with 5 µg/ml insulin, 0.5 µg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 50 µg/ml bovine pituitary extract, 10 ng/ml cholera toxin, and antibiotics (37). Only cultures of passages 2–5 were used throughout this study. The primary antibodies were used in the immunoblotting experiments. (a) anti-p53: a mouse monocular anti-human wild-type p53 (clone DO-1; amino acids 37–45 as the immunogen) from Santa Cruz Biotechnology; (b) anti-p21: a rabbit polyclonal anti-human peptide antibody (amino acids 146–164 as the immunogen) from Santa Cruz; (c) anti-Bcl-2: a mouse monoclonal anti-human peptide Bcl-2 (amino acids 41–54 as the immunogen) from either Dako (clone 124) or Calbiochem (clone 100), and a rabbit polyclonal anti-human Bcl-2 raised against a peptide corresponding to amino acids 4–21 (sc-492) from Santa Cruz; (d) anti-Bcl-Xs: a rabbit polyclonal anti-human peptide antibody (amino acids 2–19 as the immunogen) from Santa Cruz; (e) anti-Bax: a rabbit polyclonal anti-human Bax peptide antibody (amino acids 150–165 as the immunogen) from Calbiochem and a rabbit anti-human Bax peptide antibody (amino acids 43–61 as the immunogen) from Pharmingen; (f) anti-Bak: a rabbit polyclonal anti-human Bak (amino acids 82–104 as the immunogen) from Santa Cruz; (g) anti-Bad: a rabbit polyclonal anti-mouse Bad (amino acids 185–204 as the immunogen) from Santa Cruz; and (h) anti-actin: a mouse monoclonal anti-actin from ICN. The secondary antibody was either goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase. Then membranes were stripped and reprobed with different antibodies. These possibilities were tested with the following two sets of experiments. In the first, PC3 and DU145 cells were harvested, and cell lysates were prepared using the TNC lysis buffer, as described above. Five hundred µg of PC3 or DU145 cell lysates were used for immunoprecipitation experiments, as described previously (41) with some modifications. Briefly, the cell lysates in 1 ml of TNC lysis were first precleared by incubating with 75 µl protein A/G-agarose (Santa Cruz Biotechnology) at 4°C for 1 h. Then 5 µg of the monoclonal anti-Bcl-2 or isotype-matched control monoclonal antibody MOPC-21 (IgG1) were added to the precleared supernatants and incubated at 4°C for 2 h under rotating conditions. At the end, 100 µl of protein A/G-agarose beads were added, and the mixture was further incubated for 1 h at 4°C. The beads with bound antigen–antibody complexes were washed extensively: TNC lysis buffer (two times), TNC-0.1% SDS (one time), TNC-1 M NaCl (one time), and TNC lysis buffer (one time). Finally, the washed beads were reconstituted in 50 µl of 2X SDS-PAGE sample buffer [125 mM Tris-HCl (pH 6.8), 10% β-mercaptoethanol, 4% SDS, 20% glycerol, and 0.001% bromphenol blue]. After boiling, 25 µl of the precipitated sample were loaded onto a 12% SDS-PAGE, and separated proteins were transferred to nitrocellulose membrane and then blotted using the monoclonal or the rabbit polyclonal anti-Bcl-2. In the second set of experiments, 50 µg of clarified PC3 or DU145 cell lysates were incubated with 2 units of alkaline phosphatase at 37°C for 4 h. At the end, 25 µg of the protein were loaded onto a 12% SDS-PAGE, and separated proteins were used for immunoblotting with the monoclonal anti-Bcl2, as described above.

RESULTS

Expression of Apoptosis Proteins in Various Prostate Cells. We first examined the expression of various apoptosis-related proteins in different types of prostate cells. The results are plotted in Fig. 1. All cells except PC3 expressed P53, as revealed by a monoclonal antibody. Note that longer exposure of the film also resulted in the p53 band in BPH cells (see also Fig. 7). P53 expressed in the primary cells, i.e., NHP, BPH, and PCA, was shown previously to be wild-type (37). LNCaP cells also express wild-type P53 (12). PC3 cells are P53-null, therefore, no protein could be detected (Fig. 1). DU145 cells expressed the highest amount of P53 protein because the p53 gene in these cells has two mutations (codons 223 and 274), resulting in accumulation of the mutant protein (12). P21, a P53 target, was expressed in all of the cells including PC3 (Fig. 1). There appear to be some differences in terms of the protein amount of P21 in different cells (Fig. 1). When using 25–100 µg of whole-cell lysates, the Mf, 26,000 Bcl-2 protein was undetectable or, in some cases (e.g., Fig. 3B), only faintly detectable in NHP cells (Fig. 1). In similar experiments, Bcl-2 was not detected at all in BPH and PCA cells (Fig. 1; see also Figs. 7 and 9)

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in several repeat experiments, even after extended exposure. The Mr 26,000 Bcl-2 protein was detected in LNCaP cells (Fig. 1). In PC3 cells, in addition to the Mr 26,000 Bcl-2, a Mr 27,000 and a Mr 30,000 protein were also detected (Fig. 1). In Du145 cells, by contrast, only the Mr 26,000 Bcl-2 protein was detected when the membrane was probed with a polyclonal antibody (data not shown). Western blotting was also performed with three proapoptotic proteins, Bax, Bad, and Bak. All of the prostate cells expressed the Mr ~22,000 Bad (Figs. 3–7) and Mr ~25,000 Bak (Fig. 1). All prostate cells except Du145 expressed the Mr ~21,000 Bax protein, as revealed by an antibody recognizing the amino acids 43–61 of human Bax. The lack of Bax protein expression in Du145 cells was also confirmed by another polyclonal anti-Bax raised against peptide 150–165 (data not shown). Interestingly, LNCaP and PC3 cells appear to express more Bax proteins than the three primary prostate cells (Fig. 1).

The monoclonal anti-Bcl-2 used in our studies did not recognize any other Bcl-2 family proteins (Fig. 1 and data not shown). Therefore, the Mr 27,000 and Mr 30,000 bands detected by this antibody in PC3 and/or Du145 cells may represent phosphorylated Bcl-2 protein (42–46) or a new Bcl-2-related protein, such as BRAG-1, which migrates at Mr ~30,000 (47), or simply represent nonspecific reaction products. We performed two sets of experiments to differentiate among these possibilities. In the first, Bcl-2 and/or Bcl-2-related proteins were immunoprecipitated, and the precipitates were further subject to immunoblotting with the polyclonal or monoclonal anti-Bcl-2. This type of “double-shot” (i.e., immunoprecipitation followed by immunoblotting) protocol has been widely used to confirm the specificity of protein bands identified on Western blot. As shown in Fig. 2A, the Mr 30,000 band was specifically immunoprecipitated down by the monoclonal anti-Bcl-2 but not by control antibody MOPC-21, suggesting that this protein represents a Bcl-2-related protein instead of a nonspecific product. Blotting with the polyclonal anti-Bcl-2 antibody produced similar results (data not shown). Note that the status of the Mr 26,000 and Mr 27,000 proteins in this experiment was not very clear due to the proximity of these bands to the immunoglobulin light chain (Mr ~25,000; Fig. 2A). The direct use of whole-cell lysates in immunoblotting again identified the Mr 26,000, Mr 27,000, and Mr 30,000 (on the order of Mr 26,000 >> Mr 27,000 >> Mr 30,000 in terms of intensity) protein bands in PC3 cells, and Mr 26,000 and Mr 30,000 (Mr 30,000 >> Mr 26,000) protein bands in Du145 cells (Fig. 2A). Only the Mr 26,000 Bcl-2 protein was detected in A431 human epidermoid carcinoma cells (used as a control; Fig. 2A, Lane I). In the second set of experiments, whole-cell lysates from PC3 and Du145 cells were treated with alkaline phosphatase and then immunoblotted with monoclonal anti-Bcl-2. As shown in Fig. 2B, in PC3 cells, phosphatase treatment dramatically decreased the amount of the Mr 27,000 protein with a simultaneous increase in the quantity of Mr 26,000 protein, suggesting that the Mr 27,000 protein in PC3 cells is phosphorylated Bcl-2. In contrast, the level of the Mr 30,000 protein in PC3 cells was hardly affected by the phosphatase treatment, which similarly did not affect the mobility of the Mr 30,000 protein in Du145 cells (Fig. 2B). The intensity of the Mr 30,000 protein in Du145 cells appeared to decrease a small amount; however, no increased Mr 26,000 Bcl-2 protein was observed after the phosphatase treatment (Fig. 2B). Taken together, these data suggest that the Mr 27,000 protein represents the phosphorylated isoform of Bcl-2 protein, and the Mr 30,000 protein may represent a novel Bcl-2-related protein, the true molecular identity of which awaits further characterization.

Survival of NHP Cells in the Absence of Trophic Factors. As early as 24 h after the withdrawal of growth factors, NHP cells stopped proliferation, as measured by MTS assays (Fig. 3A). Thereafter, interestingly, there appeared to have a slight “bouncing-back” in the cell growth 48–72 h after culturing in the absence of trophic factors (Fig. 3A), as observed consistently in several repeat experiments. The reason for this observation was not clear. DNA fragmentation assays (Fig. 3B, inset), DAPI staining (Fig. 3C), and MTT assays (not shown) revealed that NHP cells died through apoptosis.
Increased apoptosis (i.e., positive DAPI labeling) was observed 24 h after growth factor withdrawal. On day 2, ~20% cells were apoptotic (Fig. 3, B and C, middle panel). By day 7, 60–70% cells were dead (Fig. 3, B and C, right panel). Characterization of cell growth and cell death by several different methods (i.e., MTS assays, DAPI staining, DNA fragmentation assays, and MTT) overall produced consistent results; NHP cells do not proliferate and undergo steady apoptotic death upon removal of trophic factors (Fig. 3 and data not shown). Note that the DNA ladder formation in all three primary cultures (Fig. 3; see also Figs. 6 and 8) was not as clear-cut as in established cell lines (see Figs. 10, 12, and 14). This is not surprising because apoptosis in primary cultures often is not accompanied by distinct DNA ladder formation (reviewed in Ref. 4). Western blotting was used to examine the changes of apoptosis proteins. As shown in Fig. 4, all proteins except P21 increased immediately after deprivation, as determined by densitometric scanning after normalizing the protein levels to that of actin (Fig. 5: see legend for details). The most significant increase was observed with Bcl-2 (Fig. 4 and 5A). The NHP cells expressed barely detectable levels of Bcl-2, which appeared to increase marginally by days 3 and 4 (Fig. 4). In contrast, all three proapoptotic proteins, i.e., Bax, Bad, and Bak, significantly increased with time (Figs. 4 and 5A). P53 also demonstrated a 2–3-fold increase, whereas its target, P21, did not show any corresponding changes and whose levels actually declined sharply by day 5 (Figs. 4 and 5A). Therefore, the P21 expression in NHP cells appears to be p53 independent.

Survival of BPH Cells in the Absence of Trophic Factors. Like NHP cells, BPH cells did not grow, upon growth factor withdrawal, as revealed by MTS assays (Fig. 6). In fact, BPH cells appeared to be more sensitive than NHP cells to deprivation because they demonstrated an even faster loss in the number of live cells (compare Figs. 6A and 3A). For example, ~40% of BPH cells were apoptotic (Fig. 6, B and C, middle) by day 2 compared with ~20% apoptosis in NHP cells at the same time (Fig. 3). By day 7, >80% cells were dead (Fig. 6, B and C, right panel). Western blotting revealed quite different patterns of changes in apoptosis proteins than found in NHP cells. The P53 up-regulation was most dramatic, with ~30-fold increase by day 3, after which its levels gradually decreased (Figs. 7 and 5B). The P21 level did not undergo appreciable changes and was not affected by P53 up-regulation. No Bcl-2 was detected in these cells (Fig. 7). The Bcl-XL level was also very low, which showed only a marginal increase (Figs. 7 and 5B). As in NHP cells, Bax and Bad proteins persistently increased with time; however, Bak did not show any changes (Figs. 7 and 5B).

Survival of PCA Cells in the Absence of Trophic Factors. PCA cells behaved completely differently with respect to their survival in the absence of trophic factors. Immediately (i.e., 24 h) after growth factor withdrawal, PCA cells proliferated (>2.5 fold increase in cell number) as determined by MTS assays (Fig. 8). PCA cells did not demonstrate significant (15–20%) apoptosis until days 5–6, as supported by faintly increased DNA ladder formation (Fig. 8B, inset). MTT assays (data not shown) also confirmed increased cell proliferation and rare cell death. The total number of live cells did not decrease, even by day 5 when increased apoptosis was observed (Fig. 8, B and C, middle panel), suggesting that continued cell proliferation compensated for the cell loss. By day 11, nearly the same number of cells (i.e., the same number of cells as initially input or 10,000 cells/well; see "Materials and Methods") were still alive (Fig. 8, A and C, right panel). The most significant changes in apoptosis proteins in PCA cells after trophic factor deprivation were the up-regulation of p21WAF-1 and Bcl-XL around day 4 (Figs. 9 and 5C), prior to the initiation of apparent DNA fragmentation (Fig. 8B, inset). Like NHP and BPH cells, PCA cells expressed extremely low levels of Bcl-2, which was undetectable (Fig. 9). In sharp contrast to NHP and BPH cells, all other proteins, including P53, Bax, Bad, and Bak, were not up-regulated (Fig. 9). However, interestingly, three prominent protein bands, i.e., a M, ~25,000 protein and a M, 54,000 protein, were simultaneously detected on day 4 after growth factor withdrawal, when using the monoclonal anti-P53 for the Western blotting (Fig. 9, top panel). These two novel entities of proteins were designated P25 and P54/55, respectively.

Survival of LNCaP Cells in the Absence of Trophic Factors. LNCaP cells demonstrated marginal proliferation upon serum starvation, as revealed by MTS assays (Fig. 10A). However, the cells did not...
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**Fig. 3.** Survival of NHP cells in the absence of trophic factors. A. quantitation of live cells using the MTS assays. The percentage cell number (compared to D0, which is 100%) represents means derived from three independent experiments; bars, SE. B. quantitation of apoptosis by DAPI staining. Inset, DNA fragmentation. C. micrographs showing DAPI-stained NHP cells starved for 0 (left), 2 (middle), or 7 (right) days. Apoptotic cells were brightly stained due to chromatin condensation. Note that in the middle and right panels, some apoptotic cells turned necrotic in culture and thus degraded. Therefore, an overall decreased cell number was noticed. The arrowheads in the left and middle panels illustrate apoptotic NHP cells, whereas the arrowheads in the right panel indicate very few live cells. X100.

proteins except P21 increased (2–10-fold) in expression levels upon serum starvation (Figs. 11 and 5D). Also like in NHP cells, the P21 levels decreased in a time-dependent manner, which was barely detectable by day 7 (Fig. 11). The only significant difference is that the up-regulation of Bcl-XL in LNCaP cells was not as dramatic as in NHP cells. Interestingly, the anti-P53 detected a lower band in addition to the expected M, 53,000 protein. The molecular nature of this lower band is unclear, although it might be a proteolytic product (48).

**Survival of PC3 Cells in the Absence of Trophic Factors.** PC3 cells behaved like PCA cells when deprived of serum growth factors. These cells demonstrated a steady growth, which peaked on day 3 (Fig. 12A). The cell proliferation plateaued on day 4 (Fig. 12A), when increased apoptosis (DAPI labeling and DNA fragmentation) was observed (Fig. 12, B and C, middle panel). This increased cell death clearly negated the cell proliferation; therefore, the net number of live cells did not further increase on day 4 (Fig. 12A). Starting from day 5, the number of live cells began to decrease (Fig. 12A), suggesting that, by this time, cell death (Fig. 12B) began to surpass the cell proliferation. Direct quantitation of apoptosis demonstrated that by day 8, ~60% of the cells were apoptotic (Fig. 12, B and C, right panel). This observation was consistent with the MTS measurement showing that approximately the same number of cells (i.e., 100% cell number) as initially input (i.e., 10,000 cells/well) were still alive (Fig. 12A) by day 8, considering that PC3 cells initially had a >2-fold proliferation (Fig. 12A). Western blotting examination of apoptosis proteins also revealed similar alterations as observed in PCA cells in that PC3 cells...
Fig. 5. Quantitative presentation of scanning data as determined by densitometry. The intensity of each individual protein band was first determined by scanning (41) and then normalized to the intensity of actin protein band. After normalization, the individual protein levels are plotted as "relative expression" (i.e., fold changes) compared with unstarved cells (i.e., D0 samples), the protein expression levels of which are considered as 1. A, NHP cells. B, BPH cells. The values for P53 are: 1, 1.8, 9.7, 30.9, 15.4, 17.5, and 15 from D0 to D6, respectively. C, PCA cells. Note that the data for P53 were not plotted because the P53 protein on immunoblot overlapped with the P54/55 doublet. D, LNCaP cells. E, PC3 cells. The values for Bcl-XL are: 1, 1.1, 0.9, 1.0, 1.2, and 1.1 from D0 to D7, respectively. F, DU145 cells. Bcl-2 represents the M, 30,000 Bcl-2 protein band.
lost their response in up-regulating all three proapoptotic proteins upon serum deprivation (Figs. 13 and 5E). In fact, the Bax protein actually went down dramatically by day 6 (Figs. 13 and 5E). Also similar to PCA cells, the P21 levels significantly increased by day 3 prior to the initiation of DNA fragmentation (Figs. 13 and 5E). Another aspect of the similarity between PC3 cells and PCA cells was revealed by the detection of the P54/55 doublet in PC3 cells on day 5 after serum starvation (Fig. 13). Interestingly, the P25 band detected in PCA cells was not observed in PC3 cells (Fig. 13). Different from PCA cells, PC3 cells expressed the \( M_r 26,000 \) Bcl-2 protein, the levels of which significantly increased upon serum starvation (Figs. 13 and 5E). The \( M_r 27,000 \) Bcl-2 protein levels also elevated simultaneously with the \( M_r 26,000 \) Bcl-2 protein (Fig. 13). In contrast, the Bcl-X\(_L\) protein did not change significantly (Figs. 13 and 5E).

**Survival of Du145 Cells in the Absence of Trophic Factors.** Du145 cells demonstrated the highest survival potential (Fig. 14A). Upon serum removal, these cells gradually proliferated, nearly doubling the cell number by day 3 when DNA fragmentation was first observed (Fig. 14B), suggesting that the cell proliferation exceeded cell death by apoptosis. The death rate in starved Du145 cells demonstrated a very slow kinetic (Fig. 14B), from ~10% on day 3, to ~15% on day 6 (Fig. 14C, middle), and to ~25% by day 12, at which time still about the same number (i.e., 10,000 cells/well initially plated) of cells were alive (Fig. 14C, right panel). A portion (~5%) of the Du145 cells in culture survived up to 30 days after serum withdrawal (data not shown). Western blotting of apoptosis proteins revealed that the proapoptotic proteins Bak and Bad did not undergo significant changes in their expression levels (Figs. 15 and 5F), as in

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**Fig. 6.** Survival of BPH cells in the absence of trophic factors. A, quantitation of live cells using the MTS assays. The percentage cell number (compared to DO, which is 100%) represents means derived from three independent experiments; bars, SE. B, quantitation of apoptosis by DAPI staining. Inset, DNA fragmentation. C, micrographs showing DAPI-stained NHP cells starved for 0 (left), 2 (middle), or 7 (right) days. Apoptotic cells were brightly stained because of chromatin condensation. Note that in the middle and right panels, some apoptotic cells turned necrotic in culture and thus degraded. Therefore, an overall decreased cell number was noticed. The arrowheads in the left and middle panels illustrate apoptotic BPH cells, whereas the arrowheads in the right panel indicate very few live cells. X100.

**Fig. 7.** Alterations in apoptosis proteins in BPH cells following growth factor withdrawal. Thirty \( \mu \)g of whole cell lysates prepared from unstarved BPH cells or from cells cultured in the absence of growth factors for various days were resolved on a 12% SDS-PAGE. The same membrane was sequentially probed with individual primary antibodies (see Fig. 1). Left, individual molecules. The arrowheads on the right point to the \( M_r 30,000 \) Bcl-X\(_L\) and \( M_r 22,000 \) Bad, respectively. Note the upper band in the Bad blot was incompletely deprobed Bax.
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Fig. 8. Survival of PCA cells in the absence of trophic factors. A, quantitation of live cells using the MTS assays. The percentage cell number (compared to D0, which is 100%) represents means derived from three independent experiments; bars, SE. B, quantitation of apoptosis by DAPI staining. inset, DNA fragmentation. C, DAPI-stained NHP cells starved for 0 (left), 5 (middle), or 11 (right) days. Apoptotic cells were brightly stained due to chromatin condensation. The arrowheads illustrate apoptotic PCA cells. X100.

PCA and PC3 cells. Also different from the PCA/PC3 cells, Du145 did not express Bax protein, as tested by several antibodies raised against different regions of the molecule (Fig. 15; data not shown). The most salient feature is the expression of the Mr 30,000 Bcl-2 protein in Du145 cells, the levels of which increased steadily after serum starvation (Figs. 15 and 5F). Other aspects in the changes of apoptosis proteins after serum starvation in Du145 cells were similar to those observed in PC3 cells. Specifically, P21 protein levels went up (nearly 2-fold), whereas the Bcl-XL underwent a slight increase (Figs. 15 and 5F). Also, like in PC3 cells, the P54/55 doublet was detected in Du145 cells on day 3 after serum starvation (Fig. 15). Interestingly, the P25 protein band was also detected (Fig. 15).

DISCUSSION

Recently, NHP, BPH, and PCA cells were established, and their growth properties and nutritional requirements were characterized (37). All three types of cells were diploid in karyotype, positive for both keratinocytes and PSA, and negative for ras and p53 mutations (37). When cultured in defined medium containing various growth factors, these cells demonstrated progressively accelerating growth properties; the population doubling times of NHP, BPH, and PCA cells were 51, 37, and 29 h, respectively (37). Here we compared the growth and survival properties of these primary prostate cells with those of three established prostate cancer cell lines in the absence of trophic factors.

We first characterized the expression of several apoptosis-related proteins in different types of prostate cells, from normal to malignant. All cells express P21, Bcl-XL, Bad, and Bak, and all cells except Du145 express Bax protein. However, primary cells generally express little or no Bcl-2, whereas LNCaP cells express abundant Bcl-2 protein. In PC3 and Du145 cells, a more complicated pattern of Bcl-2 expression was observed. In addition to the regular Mr 26,000 Bcl-2, PC3 cells also express two minor upper bands that migrate at Mr 27,000 and Mr 30,000, respectively. In contrast, Du145 cells

Fig. 9. Alterations in apoptosis proteins in PCA cells after growth factor withdrawal. Thirty µg of whole-cell lysates were prepared from unstarved PCA cells or from cells cultured in the absence of growth factors for various days were resolved on a 12% SDS-PAGE. The same membrane was sequentially probed with individual primary antibodies (see Fig. 1). Left, individual molecules. The arrowhead on the right points to the Mr ~30,000 Bcl-XL. Right, novel P25 and P54/55 proteins.

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treatment established the Mr 27,000 band but not the Mr 30,000 band as the phosphorylated Bcl-2 protein. Therefore, the Mr 30,000 Bcl-2 protein identified in PC3 and Du145 cells is different from the Mr 30,000 Bcl-2 in HT29 cells, which is a hyperphosphorylated form of the Mr 26,000 Bcl-2 (46). It is not yet known whether the Mr 30,000 Bcl-2 protein in PC3/Du145 cells is or is related to the newly reported Mr 30,000 BRAG-1, a Bcl-2-related protein primarily expressed in the brain (47). Both Mr 27,000 and Mr 30,000 Bcl-2 proteins are detected in PC3 and/or Du145 cells without any prior stimulation, suggesting that these proteins are endogenously expressed in both cells.

appear to primarily express the Mr 30,000 band with very low levels of the Mr 26,000 Bcl-2 protein. It is well known that Bcl-2 can undergo phosphorylation on serine residues, resulting in either Mr 27,000 Bcl-2 in multiple cell types such as leukemia, MCF-7, and PC3 cells (42-46) or Mr 30,000 Bcl-2 in HT-29 colon cancer cells (46). The Mr 30,000 Bcl-2 protein has not been reported in either PC3 or Du145 cells, even after treatment with taxol or okadaic acid (42, 43, 45). We therefore further characterized the Mr 27,000 and Mr 30,000 bands detected in PC3 and/or Du145 cells by the monoclonal anti-Bcl-2 antibody. Immunoprecipitation followed by Western blotting first established the Mr 30,000 band in both cell types as a specific Bcl-2-related protein instead of nonspecific antibody binding. Subsequently, a dephosphorylation experiment with alkaline phosphatase
in the absence of trophic factors, than in PC3/PCA/Du145 cells (summarized in Fig. 16), suggesting that cancerous prostate cells naturally possess a longer life span (or survivability) than NHP/BPH cells.

LNCaP cells, initially isolated from the lymph node metastasis of a prostate cancer patient, demonstrate a survivability between that of NHP/BPH and that of PC3/PCA/Du145 cells. These cells behave differently from other cancer cells in that they do not proliferate significantly in the absence of trophic factors. However, LNCaP cells also behave differently from NHP/BPH cells in that they do not die immediately after serum withdrawal. Instead, these cells keep a relatively constant cell number for 5–6 days, although the apoptosis is initiated at approximately day 4. The mechanism(s) for the unique survival characteristics of LNCaP cells are presently unknown. Taken together, the present data suggest that prostate cancer cells generally demonstrate longer survivability than their normal counterparts due to increased proliferative capability and decreased cell death rate. The increased cell proliferation results from the production by cancer cells of various growth factors (49), whereas the decreased death rate

Growth and Survival of Various Prostate Cells upon Trophic Factor Withdrawal. NHP and BPH cells do not grow and demonstrate the lowest survival capacity upon trophic factor withdrawal. Apoptosis is observed in these cells as early as 24 h after growth factor withdrawal. In contrast, primary (PCA) and metastatic (PC3 and Du145) carcinoma cells survive much longer, and these cells keep dividing and proliferating in the absence of trophic factors. These three cancer cell types follow very similar growth/survival patterns upon removal of trophic factors: an initial aggressive growth phase (the first 2–3 days) in which apoptosis rarely occurs; a second “countervailing” phase in which cell loss by apoptosis (taking place on days 3–4) is compensated by sustained cell proliferation; and a third “death” phase in which apoptosis surpasses the cell growth. Direct measurement of cell death by DAPI staining and MTT assays (not shown) has demonstrated a much higher death rate in NHP/BPH cells,
In this study, there appears to have a correlation between the P53 response and apoptotic sensitivity of various prostate cells. NHP, BPH, and LNCaP cells, which are the cell types sensitive to apoptosis induction by trophic factor withdrawal, demonstrated a time-dependent increase in P53 protein expression. BPH cells demonstrated the most robust (~30-fold increase by day 3) and longest up-regulation of P53, and these cells are most sensitive to apoptosis induction. In contrast, PCA cells, although expressing wild-type P53, do not demonstrate up-regulation of P53, and these cells survive much longer than NHP, BPH, and LNCaP cells (Fig. 16). Similarly, PC3 and Du145 cells, which do not express functional P53, also possess longer survivability in the absence of trophic factors. Taken together, these observations correlate an up-regulation of functional P53 with increased sensitivity of prostate cells to apoptosis induction by deprivation (Fig. 16).

Quite contrasting alterations are also seen with P21 in sensitive results from evasion of apoptosis. It is of note that PCA, one of the primary cell types used, demonstrates even higher survivability than LNCaP and PC3 cells, which are established cell lines kept in culture for many generations. Therefore, it is unlikely that any of the differences observed among these cells may have resulted from tissue culture artifacts.

Role of p53 and P21 in Prostate Cell Growth/Apoptosis upon Trophic Factor Withdrawal. p53 plays two essential roles in maintaining the cellular homeostasis: introducing “checkpoints” to arrest cell cycle; and triggering apoptosis to self-destroy those cells beyond repair (50). p53 mediates its biological functions by transcriptionally regulating (either activating or inhibiting) many of its targets, among which is p21, a CIP/KIP family cyclin-dependent kinase inhibitor. In response to genotoxic shocks, cells generally halt cell cycle progression by up-regulating P21 protein in a p53-dependent manner. Overexpression of wild-type p53 induces apoptotic death of a wide spec-
Role of Bcl-2 and Bcl-X<sub>L</sub> in Prostate Cell Growth/Apoptosis upon Trophic Factor Withdrawal

Bcl-2 and Bcl-X<sub>L</sub> are two members in the Bcl-2 family that possess anti-apoptotic functions. Many biological activities have been assigned to these proteins for their death-sparing effects: forming membrane channels; modulating mitochondria permeability transition pore; retarding cytochrome c and/or AIF apoptosis-inducing factor release from mitochondria; working as antioxidants; preventing calcium fluctuation; sequestering prosapoptotic proteins (such as Bax, Bak, and Bik); and indirectly modulating caspase activity (through CED-4), cell cycle (through p53BP-2), NF-AT transcriptional activity (through calcineurin), or the death-promoting activity of Bad (through Raf-1: Refs. 55 and 56). The precise mechanism(s) for the anti-death effects of Bcl-2/Bcl-X<sub>L</sub> is still unknown and most likely is cell type and inducer dependent. Bcl-2 has been shown to be phosphorylated by Raf-1 and some other protein kinases, and this phosphorylation has been hypothesized to inactivate the cytoprotective effect of Bcl-2 (42–46). In addition, both Bcl-2/Bcl-X<sub>L</sub> and P21 could be inducing a cell cycle arrest because the peak level of P21 expression is unrelated to or independent of the P53 status, which is consistent with data showing that P21 expression can be p53-independent (50, 51); (b) the involvment of P53 and P21 in apoptosis of prostate cells is independent of each other, as reported by others in oncogene-mediated fibroblast apoptosis (52); and (c) increased P21 expression is protective for prostate cancer cells, as observed in other cell systems (53, 54). The fact that P21 levels go up before apoptosis suggests a causal rather than a coincidental relationship between the two phenomena. The increased P21 could be inducing a cell cycle arrest because the peak level of induction of this protein also coincides with the time when cells stop proliferation. It is known that dividing cells are much more prone to apoptosis induction (55); therefore, it is quite likely that the increased P21 will put a “brake” on the proliferative machinery in prostate cancer cells to avoid “mitotic catastrophe” (55).

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Role of Bax, Bad, and Bak in Prostate Cell Growth/Apoptosis upon Trophic Factor Withdrawal. In contrast to Bcl-2 and Bcl-X_L, proapoptotic proteins Bax, Bad, and Bak demonstrate very distinct alterations in their expression levels that highly correlate with the sensitivity to apoptosis induction in individual cell types (Fig. 16). Specifically, upon growth factor withdrawal, all sensitive cells, i.e., NHP, BPH, and LNCaP, demonstrate a time-dependent up-regulation of these proteins. In contrast, long-living cells (PCA, PC3, and Du145) invariably demonstrate a loss of this up-regulation response. More dramatically, PC3 cells demonstrate a gradual decrease, and Du145, the most resistant cells, demonstrate a complete loss in Bax expression. The Bax/Bad/Bak protein expression appears to be correlated with the p53 functionality; their levels are increased in cells with wild-type p53 (NHP, BPH, and LNCaP), and their levels show no increase or show decrease or loss in cells with no or mutant p53 (PC3 and Du145). PCA cells, although expressing functional p53, somehow do not up-regulate this protein in response to deprivation and therefore resemble PC3 and Du145 cells in terms of p53 functionality.

Role of Novel Proteins in Prostate Cell Growth/Apoptosis upon Trophic Factor Withdrawal. In addition to the novel M, 30,000 Bcl-2 protein detected in Du145 cells, several other proteins, specifically P25 and P54/55 doublet, are also detected in PCA, PC3, and Du145 cells. These proteins appear together at the same time and can be detected by at least two unrelated monoclonal antibodies, i.e., monoclonal anti-P53 (in PCA cells; Figs. 9 and 10) and monoclonal anti-Bcl-2 (in PC3 and Du145 cells; Figs. 13 and 15). Based on these characteristics and their apparent molecular weights, these proteins appear to be immunoglobulin heavy (M, $\sim$55,000) and light (M, $\sim$25,000) chains (see also Fig. 2). Why these “immunoglobulin molecules” are up-regulated is unclear. However, the fact that they are only up-regulated in resistant cells (PCA, PC3, and Du145) and their regulation follows a distinct pattern (i.e., around the time when cells initiate apoptotic programs) point to an intriguing possibility that they might be causally involved in extending the survival of prostate cancer cells.

Synopsis. The present study uses a simple model to compare the growth/survival characteristics of normal versus cancerous prostate cells cultured in the absence of trophic factors. Fig. 16 summarizes the sequential molecular events that occur as these cells progress from a sensitive to a resistant stage with respect to apoptosis induction. NHP and BPH cells express wild-type p53 and little/no Bcl-2. In response to growth factor withdrawal, these cells stop proliferation and rapidly up-regulate p53, which may then up-regulate proapoptotic proteins Bax/Bad/Bak. Cells rapidly enter an apoptotic phase. In contrast, prostate cancer cells, upon deprivation, still proliferate aggressively due to autocrine production of various growth factors. These cells (except PCA) endogenously express much higher levels of Bcl-2. They express either mutant p53 (PC3 and Du145) or wild-type p53, which somehow does not respond to starvation by up-regulating its expression (PCA), leading to the loss of up-regulation or decrease of proapoptotic proteins. Furthermore, cancer cells such as Du145 even completely delete a proapoptotic protein (i.e., Bax). At the same time, these cells up-regulate P21, which could induce cell cycle arrest at an appropriate time (because proliferating cells are generally more sensitive to apoptosis induction; Ref. 55), and Bcl-2, which by itself could extend cell survival. Last but not least, the long-living cells express some novel immunoglobulin or immunoglobulin-like proteins that might also help maintain the cell survivability. Cells with intermediate life span, i.e., LNCaP, demonstrate a variety of characteristics lying between the above two cell types. Sustained proliferation, together with multiple molecular mechanisms to evade apoptosis, leads to significantly extended survivability of prostate cancer cells, compared with their normal counterparts (Fig. 16).
APOPTOSIS AND PROSTATE CANCER CELL SURVIVAL


Extended Survivability of Prostate Cancer Cells in the Absence of Trophic Factors: Increased Proliferation, Evasion of Apoptosis, and the Role of Apoptosis Proteins

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