

Enhanced Redundancy in Akt and Mitogen-activated Protein Kinase-induced Survival of Malignant *versus* Normal Prostate Epithelial Cells

Aarti R. Uzgare and John T. Isaacs

Anti-Cancer Drug Discovery/Development Program, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, Maryland

ABSTRACT

Activation of the downstream akt and mitogen-activated protein kinases is associated with development and progression of prostate cancer to the lethal androgen-independent state. However, the causal role of these downstream kinases in androgen-independent prostate cancers is unknown. In this study, activation and requirements of akt and mitogen-activated protein kinase (erk, p38, and jnk) signaling for the survival and proliferation of five malignant human cell lines encompassing the spectrum of androgen-independent prostate cancers was compared with the activation and requirements in normal prostate epithelial cells. Using Western blotting with phospho-antibodies, we detected differential activation in exponentially growing, growth factor-deprived, and restimulated cultures of malignant *versus* normal cells. The inhibition of erk, p38, jnk, and akt with U0126, SB203580, SP600125, and Akt inhibitor, respectively, document that normal cells require simultaneous erk and jnk signaling for survival, plus akt signaling for proliferation. In malignant cells, however, only jnk inhibition as monotherapy produces a consistent apoptotic response, although the combinatorial inhibition of jnk, erk, p38 plus akt results in statistically enhanced apoptosis. These results demonstrate that prostate cancer progression to a lethal androgen-independent state involves the acquisition of an enhanced redundancy in downstream survival signaling.

INTRODUCTION

Androgen is the major growth and survival factor for the normal prostate via a paracrine interaction between the stromal and epithelial compartment (1). In stromal cells, androgen occupancy of nuclear androgen receptors activates the synthesis and secretion of peptide growth and survival factors known as andromedins. These andromedins diffuse across the basement membrane into the basal layer of the epithelial compartment, in which they bind to cognate receptors in androgen receptor-negative transit-amplifying cells (TA), inducing the appropriate signaling pathways to regulate their survival and proliferation. These TA cells are derived from stem cells and are the subset of proliferating epithelial cells within the prostatic glands that give rise to prostatic adenocarcinomas (1, 2). During prostatic carcinogenesis, there is a shift in the androgen axis from stromal cell-dependent paracrine signaling to an autocrine pathway in which occupancy of the androgen receptor within the cancer cells themselves directly stimulates their survival and proliferation (3). Thus, androgen ablation therapy induces the apoptotic death of these androgen-dependent cells, producing an initial response (4). Unfortunately, this positive clinical response is nearly universally followed by a relapse to a refractory state in which the cancer cells continue to survive and proliferate despite a low, circulating androgen environment. Such a relapse documents that these cancer cells have acquired alternative

ways of activating survival and proliferative pathways without requiring physiological levels of circulating androgens (5, 6). This involves either alternative mechanisms for activating the same pathway(s) required by normal prostatic epithelial cells (PrECs) or initiating new ectopic pathways (6). Resolving this issue is more than academic, because at present, there is no effective therapy for this hormone-refractory state, making such progression a lethal event. Thus, identification of the signal transduction pathways responsible for the survival and proliferation of hormone-refractory prostate cancer cells is critical for rational drug development.

As a starting point for such identification, examination of the role of mitogen-activated protein kinases (MAPKs; *i.e.*, erks, jnks, and p38) and of akt is appropriate because they are key downstream mediators in the signaling cascades activated via trophic ligand receptor interactions for survival and proliferation in other cell types (7, 8). In addition, these kinases are expressed in both normal and malignant PrECs (9, 10). The activation of akt is significantly increased in primary human tumors, particularly those representing late-stage cancers; this is associated with the loss of PTEN and p27 kip protein expression (10–14). The activation of erk and p38 is detectable in premalignant lesions and/or early-stage cancers and, hence, may play a role in the genesis or early progression of prostatic disease (10, 11, 15–17). Although elevated levels of phosphorylated (*i.e.*, activated) erk have been reported in advanced disease (16, 18), additional studies indicate that erk activity declines with disease progression (10, 11, 17) and that constitutive erk activity inhibits prostate cancer proliferation (19). Activated jnk is detectable in the nuclei of basal epithelial (*i.e.*, TA) cells in the normal prostate, and there is a quantitative increase in activated (*i.e.*, nuclear) jnk in prostatic cancer cells (9, 18).

Although these correlative studies are important, they do not resolve the causal *versus* associated nature of the activities of akt and MAPK in androgen-independent prostate cancer cells. To evaluate the causal role of these signaling pathways, appropriate *in vitro* test systems are required. As an appropriate test system, we characterized the role of akt and MAPK pathways in the *in vitro* survival of normal prostatic transit-amplifying cells. This is because TA cells, although being ligand (*i.e.*, androgen) independent, do require andromedin signaling for their *in vitro* survival and proliferation (20) and also because their transformed progeny give rise to prostate cancer (2). These results were compared with those from a series of five androgen-independent prostate cancer cell lines to identify whether similar or alternative signaling pathways are consistently involved with malignant *versus* normal prostatic cell survival and proliferation.

MATERIALS AND METHODS

Cell Culture and Reagents. Normal PrECs (Clonetics, Inc., Walkersville, MD) and 6E (3) were maintained (up to a maximum of six passages) in prostate epithelial basal medium supplemented with bovine pituitary extract, epidermal growth factor, insulin, transferrin, hydrocortisone, retinoic acid, epinephrine, tri-iodothyronine and gentamicin-amphotericin solution (Clonetics Inc.). All of the malignant cell lines were cultured in 10% fetal calf serum (FCS; Hyclone, Logan, UT) containing RPMI 1640 (Invitrogen, Carlsbad, CA) except for LAPC-4, which was grown in serum and 1 nmol/L R1881 (Perkin-Elmer, Wellesley, MA) containing Iscove's medium (BioFluid, Rockville,

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Requests for reprints: John Isaacs, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, CRB-1M41, Baltimore, MD 21231. Phone: 410-955-7777; Fax: 410-614-8397; E-mail: isaacjo@jhmi.edu.

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MD). Typically, cells were seeded at a density of 1,500 malignant cells per well or 3,200 normal cells per well in 96-well plates, or 200,000 cells per well in 6-well plates in complete (growth factor-containing) media. For starvation/growth factor deprivation experiments, complete media was replaced with basal media (*i.e.*, serum-free media in the case of malignant cells or supplement-free media in the case of normal cells).

Stock solutions of 20 mmol/L U0126 (Promega Corp., Madison, WI), 10 mmol/L SB203580 (LC Labs, Woburn, MA), 100 mmol/L SP600125 (Calbiochem, San Diego, CA), and 20 mmol/L Akt inhibitor (Calbiochem) were prepared in DMSO and were stored as per manufacturer's instructions. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Casodex was kindly provided by AstraZeneca Pharmaceuticals (Cheshire, UK).

Cell Growth Assays. Live cells were quantitated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay from Promega Corp.), as described previously (20). Cell numbers were determined by extrapolation from standard curves of absorbance *versus* cell number. The number of cells estimated after each treatment was normalized to the starting cell number (1,500 malignant cells per well or 3,200 normal cells per well) and represented as 100%. Data shown are means ± SEM of three independent experiments.

Western Blotting. Western blots were performed with 50 µg of total protein except in the detection of p38, for which 100 µg were used. Primary antibodies to phospho-erk1/2 (E10 monoclonal), phospho-p38MAPK (polyclonal), phospho-Akt^{ser473} (polyclonal), total erk1/2 (polyclonal), total p38MAPK (polyclonal), and total akt (polyclonal) were purchased from Cell Signaling Technology (Beverly, MA); p27 kip and p21cip monoclonal antibodies were purchased from BD Biosciences (San Diego, CA); Rb (C-15), p53 (FL-393), phospho-c-jun (serine 73-R), total c-jun (H-79), and androgen receptor (N-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); PTEN (clone 6H2.1) from Cascade Bioscience (Winchester, MA); and involucrin (clone 545) and transglutaminase II (clone TG-100) from Neomarkers, Inc. (Fremont, CA). Blots were routinely stripped in buffer containing 1% β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) for 1 hour at 37°C and reprobed as mentioned in the figure legends. Secondary horseradish peroxidase-conjugated antibodies and chemiluminescent detection reagents (ECL) were purchased from Amersham Biosciences Corp., Piscataway, NJ. Quantitative estimation of band intensity was determined by densitometry with Kodak Digital Science software. The ratio of the amount of phosphorylated protein to the amount of total protein was calculated in each case.

Determination of Percentage of Proliferating Cells. Cytospun (100 µl of 2 × 10⁵ cells/ml suspension) cultures were fixed in 3% paraformaldehyde (4°C for 30 min) and were stained with an antibody directed against Ki67 (Immunotech Inc., Marseille, France) at a dilution of 1:50, as described previously (3). The percentage of cells in S phase was assayed by bromodeoxyuridine (BrdUrd) incorporation in the 5-bromo-2'-deoxyuridine Labeling and Detection Kit II from Roche (Indianapolis, IN), as described by the manufacturer. Mean results were obtained from three experiments, each being scored from a minimum of three fields.

Determination of Percentage of Apoptotic Cells. Seventy percent-methanol-fixed cells were rehydrated and stained with a 1.5 µg/ml concentration of 4',6-diamidino-2-phenylindole (DAPI) in phosphate buffer saline (Vector Laboratories, Burlingame, CA). The stained cells were observed under a Zeiss UV-fluorescent microscope for apoptotic morphology, as described previously (21). Mean results were obtained from three experiments, each being scored from a minimum of three fields.

Time-Lapse Videomicroscopy. T-25 flasks seeded with 6 × 10⁵ cells were imaged using phase contrast optics and were evaluated for proliferation and apoptosis, as described previously (21).

RESULTS

Characteristics of the Normal and Malignant Human Prostatic Epithelial Cells Used. In serum-free, growth factor-supplemented medium, cultures of TA epithelial cells can be routinely established from normal prostatic tissue (20). These cultures have a 48-hour doubling time and can be serially passaged in serum-free defined medium 7 to 10 times before undergoing proliferative quiescence.

When Western blotting and reverse transcription-PCR are used with early passage cells (*i.e.*, before passage 7), the cells express detectable levels of PTEN, Rb, p53, p63, and p21cip proteins but do not express detectable levels of p27kip mRNA or protein, which is characteristic of normal transit-amplifying PrECs (20). In early passage (*i.e.*, before seven passages), these normal prostate epithelial cells do not transcribe reverse transcription-PCR detectable levels of androgen receptor mRNA and, thus, do not express detectable levels of any of the prostate differentiation markers [*i.e.*, prostate-specific antigen (PSA), human kallikrein 2 (hK2), prostate-specific membrane antigen (PSMA)] mRNAs (20), the latter being characteristic of secretory luminal cells (1). Although these early passage cells do not express androgen receptor and, thus, do not require androgen for their optimal growth, a high-growth fraction (as assayed by Ki67 expression) and high S-phase fraction (measured by BrdUrd incorporation) does require the addition of growth factors in the medium to induce downstream signaling cascades for proliferation and survival (Table 1).

For comparison with normal human PrECs, we used a series of five human prostatic cancer cell lines. These malignant lines were chosen because they are derived from local disease, soft tissue, or bone metastases and vary widely in phenotypic and genotypic characteristics mimicking the range of behavior of prostate cancers in the clinic (Table 2). In addition, all of these cancer cell lines are androgen independent (*i.e.*, do not require androgen in the medium for their survival and proliferation) and, thus, are the type of cells that are ultimately lethal to prostate cancer patients (1). Although androgen independent, two of these lines (LAPC-4 and CWR22Rv1) still retained a limited sensitivity to androgens as documented by the ability of the anti-androgen Casodex, at concentration 10 µmol/L, to inhibit proliferation when added to the optimal growth medium, even though it did not induce apoptotic death of either cell line (Table 3). In contrast, the remaining lines [*i.e.*, in late passage (>100 passages) LNCaP (termed LNCaP-LP), PC-3, and DU-145] were androgen independent and had no response to Casodex (Table 3). In addition, because normal and malignant prostate epithelial cells both produced high-growth fraction (*i.e.*, >60% Ki67-positive) cultures *in vitro* (Table 1), valid comparisons can be made about the importance of specific downstream kinase signaling in their growth regulation.

Table 1 Growth response of normal and malignant prostate epithelial cells to 3 days of growth factor deprivation

Cell line	Growth factor status*	Growth fraction (% Ki67 positive)	S-phase fraction (% BrdUrd positive)
PrEC	+	65 ± 6	38 ± 2
	-	11 ± 6† (17)	5 ± 2† (13)
CWR22Rv1	+	75 ± 4	35 ± 8
	-	27 ± 12† (36)	8 ± 1† (23)
LAPC-4	+	75 ± 6	21 ± 4
	-	15 ± 3† (20)	7 ± 2† (33)
LNCaP-LP	+	89 ± 6	51 ± 6
	-	8 ± 3† (9)	4 ± 1† (8)
PC-3	+	75 ± 7	34 ± 16
	-	13 ± 1† (17)	4 ± 1† (12)
DU-45	+	97 ± 1	30 ± 6
	-	30 ± 4† (31)	14 ± 3† (47)

Note. Data shown are mean ± SEM. Values in parentheses are the percentage of the value for that particular cell line without growth factor relative to the value for that cell line in the presence of growth factor.

* Growth factor starvation (-) or stimulation (+) was achieved by serum deprivation or addition in the case of malignant cells and via supplements in case of normal cells.

† P < 0.05, difference between + versus - growth factor value for that particular cell line.

Table 2 Characteristics of human prostate cancer cell lines utilized for comparison with normal prostatic epithelium

Cell line	Site of origin	Expression of indicated protein								
		PTEN	p53	Rb	p21cip	p27kip	AR	PSA ng/10 ⁷ cells	hK2 ng/10 ⁷ cells	PSMA activity pmol/min/mg
CWR22Rv1	Primary cancer	++ (wt)	+ (wt)	+++ (wt)	+++	+++	+++ (m)	12.0 ± 3.0	11.0 ± 3.0	21.8 ± 1.7
LAPC-4	Lymph node metastasis	+++ (wt)	+++ (m)	+++ (wt)	–	+	+++ (wt)	8.5 ± 1.0	0.6 ± 0.2	1.0 ± 0.1
LNCaP-LP	Lymph node metastasis	– (m)	+ (wt)	+++ (wt)	+	+	+++ (m)	67 ± 15	22 ± 6	29.0 ± 2.6
PC-3	Bone metastasis	– (m)	– (m)	+++ (wt)	–	+	– (wt)	<0.1	<0.1	<0.1
DU-145	Brain metastasis	+ (wt)	+++ (m)	– (m)	–	++	– (wt)	<0.1	<0.1	<0.1

NOTE. +++, high level of expression; ++, moderate level of expression; +, low but detectable expression; –, no detectable expression by Western blotting. Wild-type (wt) and mutated (m) genes are based on ref. 13 (for PTEN), ref. 38 (for p53 and AR), and ref. 39 (for Rb). Serum prostate-specific antigen (PSA) and human kallikrein 2 (hK2) levels and prostate-specific membrane antigen (PSMA) activity are as reported in ref. 40. Data shown are mean ± SEM.

Level of Akt and MAPK Activation in Growth Fraction-Matched Cultures of Normal versus Malignant Prostate Cells.

Activation of akt and the MAPKs (*i.e.*, erk, jnk, and p38) was evaluated in high-growth fraction cultures of commercially obtained (*i.e.*, PrEC) and in-house-derived (*i.e.*, 6E) normal TA cells. To do this, we carried out Western blot analyses using phosphorylation-specific antibodies to detect the level of active (*i.e.*, phosphorylated) erk, p38, akt, and c-jun [to detect jnk activity (22)] versus phosphorylation-independent antibodies to quantitate the total level for each of the kinases (Fig. 1). These results documented that proliferating normal prostate TA cells had detectable steady-state levels of active erk1 and 2, p38, c-jun, and akt. These results were compared with those obtained using the five prostatic cancer cell lines. To do this, we normalized the results to the level of kinase activity observed in normal prostatic transit-amplifying (PrEC) cells (Fig. 1) under optimal growth conditions (*i.e.*, in the presence of growth factors) as determined by Ki67 expression (Table 1).

Under such optimum conditions of growth factor-induced signaling, normal PrECs (PrEC and 6E) displayed higher levels of erk 1 and 2 activity compared with all of the malignant cell lines (Fig. 1A), even though they all had comparable growth and S-phase fractions (Table 1). In PC-3 cells, p38 activity was comparable with that in normal PrECs, although p38 activity was reduced in the other four malignant cell lines (Fig. 1B). Jnk activity, as assayed by phospho-c-jun levels, was significantly increased in all of the malignant lines, compared with normal cells (Fig. 1C). Akt activation was higher in the PTEN mutant LNCaP and PC-3 cells, as compared with normal PrECs (Fig. 1D). Akt activity was lower, however, in DU-145 and CWR22Rv1 cells (*i.e.*, both PTEN wild-type) and equal in LAPC-4 cells than in normal PrECs (Fig. 1D). The above results documented that there

were consistent differences in the steady-state levels of activation of the various MAPKs in malignant (lower erk and p38 activity, and higher jnk activity) versus normal prostate cancer cells, even though the cells were matched for growth and S-phase fractions.

Differential Response of the Akt and MAPKs to Growth Fraction Deprivation in Normal versus Malignant Prostate Cells. The high rate of proliferation of normal TA cells was rapidly decreased ($P < 0.05$) after removal of the exogenous growth factors (Table 1). After a 3-day period of growth factor withdrawal, the number of viable normal cells remained constant (20), although the growth fraction (*i.e.*, the percentage of Ki67 positive) and S-phase fractions (*i.e.*, the percentage of BrdUrd incorporation positive) decreased ($P < 0.05$) by more than 70% (Table 1). At this 3-day deprivation time point, akt activation was decreased by 2- to 3-fold, phosphorylated p38 and jun levels (*i.e.*, jnk activity) remained unchanged, and phosphorylated erk 1/2 levels were twice as high, as assayed by the densitometric levels of phosphorylated protein normalized to that of β-actin (Fig. 2). Expression of p21cip and p27kip in these growth factor-deprived normal cells was tested, because these molecules can inhibit entrance into the cell cycle by their binding to cdk2/cyclin E and other cdk/cyclin complexes (23). Whereas such growth factor deprivation decreased the proliferation of PrECs (Table 1), it did not induce any change in p21cip protein nor induce p27kip protein expression (Fig. 2). These results further suggested that survival of normal TA cells was provided by cell-attachment-dependent signaling, although proliferation requires additional growth factor-dependent signaling.

Like the situation for normal PrECs, the growth and S-phase fraction for each of the malignant prostate lines was high in the presence of growth factors (Table 1). In contrast to normal prostatic cells, however, p27kip was expressed by all of the malignant prostatic cell lines, although only two of five lines (*i.e.*, CWR22Rv1 and LNCaP-LP) expressed p21cip during high-growth-fraction growth in the presence of growth factors (Fig. 2). To deplete the levels of autocrine growth factors, malignant cell lines were washed and fresh medium that did not contain androgen or serum was replaced daily. After 3 days of such growth factor deprivation, the growth and S-phase fraction of each of the malignant lines was significantly ($P < 0.05$) reduced (Table 1) with no indication of an enhancement in apoptosis as assayed by increased DAPI staining or decreased viability using MTT assays (data not shown). Thus, like the situation for normal prostatic cells, malignant cell attachment are sufficient to induce survival signaling, although continuing to require growth factor input for proliferation. Unlike the situation for normal prostate cells, however, associated with this decrease in growth fraction was an

Table 3 Characteristic growth response of human prostate cancer cell lines to anti-androgen

Cell line*	No. of cells after 7 days with indicated treatment	
	Optimal medium†	Optimal medium + 10 μmol/L Casodex
LAPC-4	21,311 ± 387‡	9,876 ± 871
CWR22Rv1	39,404 ± 7,511‡	20,175 ± 1,975
LNCaP-LP	189,644 ± 1,927	195,672 ± 2,141
PC-3	161,208 ± 10,603	175,145 ± 2,581
DU-145	243,589 ± 2,423	238,417 ± 1,796

Note. Data shown are mean ± SEM.

* Two thousand viable cells were inoculated at time 0.

† Optimal growth medium contains 10% FCS only except for LAPC-4, which additionally contains 1 nmol/L R1881.

‡ $P < 0.05$, compared with cell number after growth in medium plus 10 μmol/L Casodex.

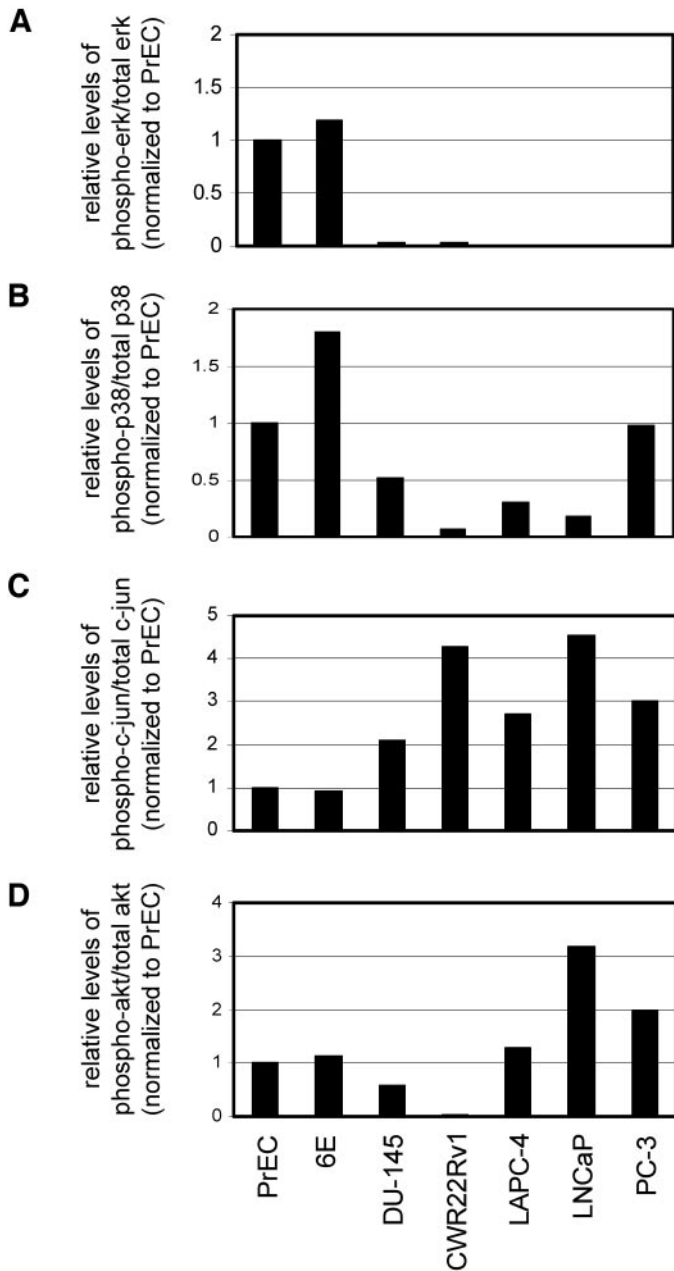


Fig. 1. The activity of erk, p38, jnk, and akt in exponentially growing cultures of normal *versus* malignant prostate cells. Growing cultures of normal epithelial (PrEC, 6E) and malignant (DU-145, CWR22Rv1, LAPC-4, LNCaP, PC-3) prostate cells were harvested, lysed, and subjected to Western blotting. Blots were probed with antibodies against phosphorylated (phospho-) erk1/2, p38MAPK, c-jun, and akt, then were stripped and re-probed with anti-erk1/2, anti-p38, anti-c-jun (for jnk activity), or anti-akt to determine total levels of each protein. Densitometric estimation of activated erk (A), p38 (B), c-jun for jnk activity (C), and akt (D) was calculated as the ratio of phosphorylated protein to total protein. The activation levels in malignant cells are shown relative to that in PrECs.

up-regulation in p27kip protein in growth factor-deprived cultures for all of these malignant lines, although p21cip expression was enhanced only in LNCaP-LP and PC-3 cells (Fig. 2). In CWR22Rv1 cells, p21cip was expressed but it did not increase after growth factor removal (Fig. 2).

Also, unlike the situation for normal prostate cells, 3-day growth factor deprivation did not result in an increase but, instead, in a decrease in erk 1 and 2 activity in all of the malignant lines except PC-3. Growth factor deprivation increased p38 activation in LNCaP-LP, LAPC-4, and PC-3 cells but decreased it in DU-145 cells, with

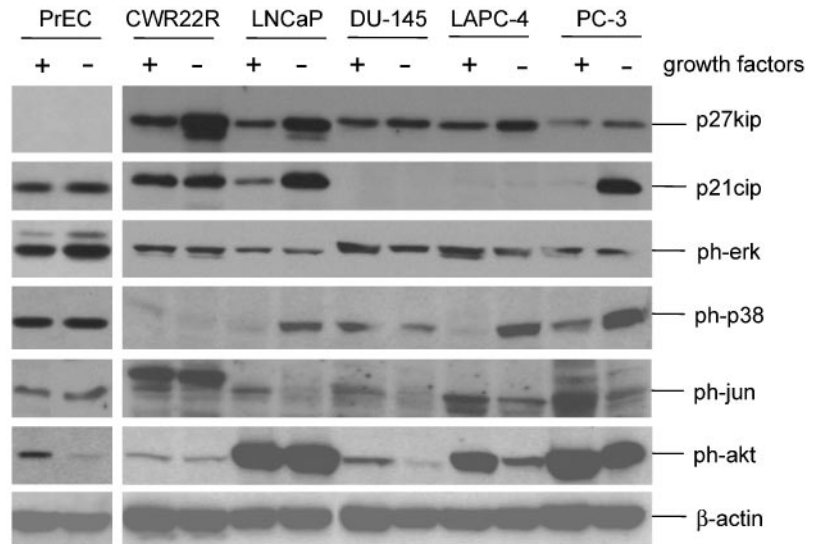
little expression in CWR22Rv1 cells (Fig. 2.) As in normal cells, akt activation is lowered by 2- to 4- fold in PTEN wild-type, CWR22Rv1, DU145, and LAPC-4 cells. Changes in akt phosphorylation were not detectable in PTEN loss of function LNCaP-LP and PC-3 cells (Fig. 2). Unlike normal cells, jnk activation, as assayed by phospho-c-jun, was decreased by growth factor deprivation in all of the malignant cells (Fig. 2). These results demonstrated that although growth factor deprivation did not induce apoptosis, it did reduce overall cell proliferation equally in normal and malignant cells (Table 1). This deprivation had differential effects on erk, p38, and jnk activity (Fig. 2; *i.e.*, increased erk, decreased akt activity in normal cells *versus* increased p38, and decreased erk, jnk, and akt activity in malignant cells).

Kinetics of Akt and MAPK Activation during Cell Cycle Progression in Normal *versus* Malignant Prostate Cells. These previous results raised the issue of whether differences in regulation of these activities occurs when cells deprived of growth factors for 3 days are induced to reenter the cell cycle after growth factor replacement. For these studies, CWR22Rv1, LNCaP-LP, and DU-145 lines were used, and the results were compared with those from normal PrECs during growth factor deprivation/restimulation-induced cell cycle recruitment. Under conditions of growth factor deprivation, erk, p38, and jnk activity remained high in normal PrECs, and these levels were not changed by growth factor restimulation (Fig. 3A and B). In contrast, erk and p38 activities were low during serum deprivation and were transiently enhanced on restimulation of malignant cells with serum (Fig. 3A and B). Densitometric quantitation of Western blots indicated that erk was maximally activated, compared with the growth factor-deprived cultures, by 29-fold ($P < 0.05$) in CWR22Rv1 at 30 minutes of restimulation, by 7-fold ($P < 0.05$) in LNCaP-LP at 30 minutes of restimulation, and by 8-fold ($P < 0.05$) in DU-145 cells at 10 minutes of re-exposure to serum. The maximal erk activation observed in restimulated CWR22Rv1 cells was transitory and monophasic, because no additional peaks of activation were detected at various time points (2, 3, 4, 5, 6, 7, 8, 22, 23, and 24 hours) during restimulation (data not shown). This was consistent with an enhanced erk activity in early G₁ phase of cell cycle. An additional peak of erk activation was observed in DU-145 cells after 24 hours of re-exposure to growth factors (Fig. 3A). Because the doubling time of these cells in the presence of growth factors was 23 hours, this peak was consistent with activation in the G₁ phase of the next replication cycle. Transient G₁ phase activation of p38 was also observed in all of the malignant cell lines tested [Fig. 3B; *i.e.*, by 37-fold ($P < 0.05$) in CWR22Rv1, by 28-fold ($P < 0.05$) in LNCaP, and by 3-fold ($P < 0.05$) in DU-145 cells] within 10 minutes of restimulation (*i.e.*, during early G₁ phase of the cell cycle). Growth factor replacement also resulted in a rapid increase in akt activity in the malignant cells; this was not a transient but a sustained 2- to 3-fold elevation over the 24-hour period (Fig. 3C).

These results documented that, whether in cell cycle or not, normal prostate epithelial cells chronically maintain MAPK activity although akt activity is high only when these cells are in cycle. In contrast, malignant prostate cells transiently activate MAPKs during recruitment into the G₁ phase of cell cycle but chronically maintain elevated akt activity during all phases of the cell cycle. These results suggest that akt and MAPK activities effect proliferation and survival of normal and malignant prostate cells differently. To test this possibility directly, we used pharmacological agents to inhibit akt and MAPK activation in normal and malignant prostate cells.

Dose Response Requirements for the Specific Inhibition of Akt and MAPK Signaling. In these studies, U0126 was used as the inhibitor of MEK1/2 (the upstream activator of the erk), to prevent activation of erk1 and 2 (24) although SP600125 was used as the inhibitor of jnk (25). SB203580 was used as the inhibitor of p38

Fig. 2. Expression of p27kip, p21cip, MAPKs, and akt in normal versus malignant prostate cells maintained with and without growth factors. Western blots demonstrating expression of p27kip, p21cip, phosphorylated erk (*ph-erk*), p38 (*ph-p38*), c-jun (*ph-jun*), and akt (*ph-akt*), and β -actin in normal (*PrEC*) and malignant (*CWR22Rv1*, *LNCaP*, *DU-145*, *LAPC-4*, *PC-3*) cells cultured in the presence (+) or absence (-) of growth factors. Growth factor stimulation or deprivation was achieved by the addition or removal of 10% FCS in the case of malignant cells and by the addition or removal of supplements in the case of normal cells. (Note from the authors: all lanes within a Western blot originate from a single experiment and a single scan of the film.)



MAPK (26). A thrice-modified phosphatidylinositol analogue was used as the akt inhibitor AIN to prevent translocation of akt to the membrane and, hence, activation by 3-phosphoinositide dependent kinase-1 and -2 (27). To develop appropriate regimens for inhibiting the individual MAPKs and akt effectively in prostate epithelial cells,

we performed preliminary dose-response studies using CWR22Rv1 cells as a validation system. U0126 successfully inhibited the activation of erk 1/2 in response to growth factor restimulation (Fig. 4A and B). At a concentration of 10 μ mol/L, U0126 diminished erk activity to a level even lower than that in growth factor-deprived cells (*i.e.*, no

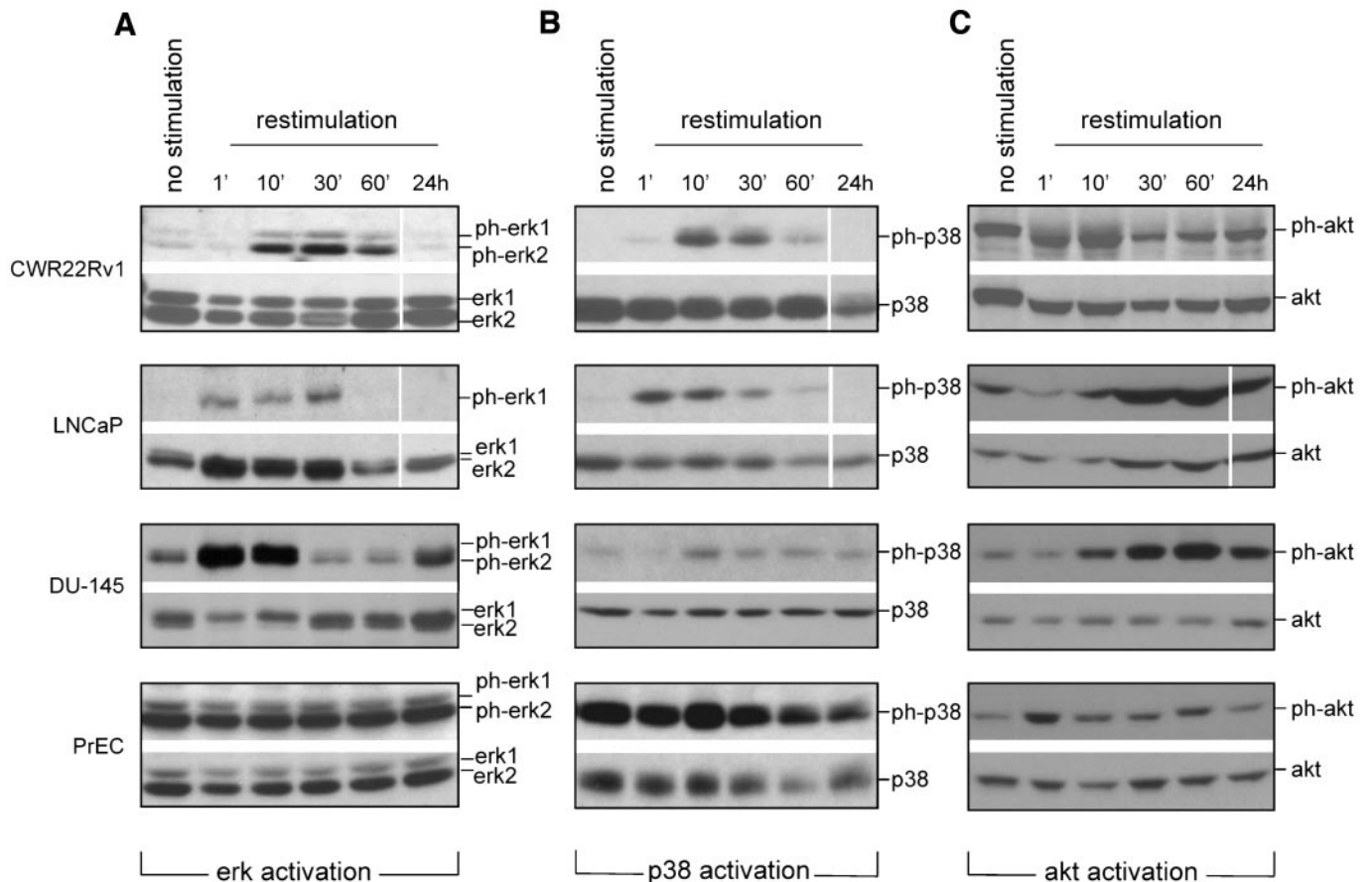


Fig. 3. Activation of erk, p38, and akt during growth factor restimulation in normal versus malignant cells. Exponentially growing cultures of malignant (*CWR22Rv1*, *LNCaP*, *DU-145*) and normal (*PrEC*) prostate cells were grown in the absence of growth factors for 3 days (no stimulation) and subsequently were restimulated with growth factors for defined periods of time [1 minute (1'), 10 minutes (10'), 30 minutes (30'), 60 minutes (60'), 24 hours (24h)]. Growth factor stimulation or deprivation was achieved by the addition or removal of 10% FCS in the case of malignant cells and of supplements in the case of normal cells. Cell lysates were electroblotted and probed with antibodies against phosphorylated erk1/2 (*ph-erk1*, *ph-erk2*; A), p38 (*ph-p38*; B), and akt (*ph-akt*; C). Respective blots were stripped and were reprobbed with total erk1/2 (A), total p38 (B) and total akt (C) as indicated. (Note from authors: all lanes within a Western blot are derived from a single experiment and scan and have been edited only to maintain a uniform order in the compilation.)

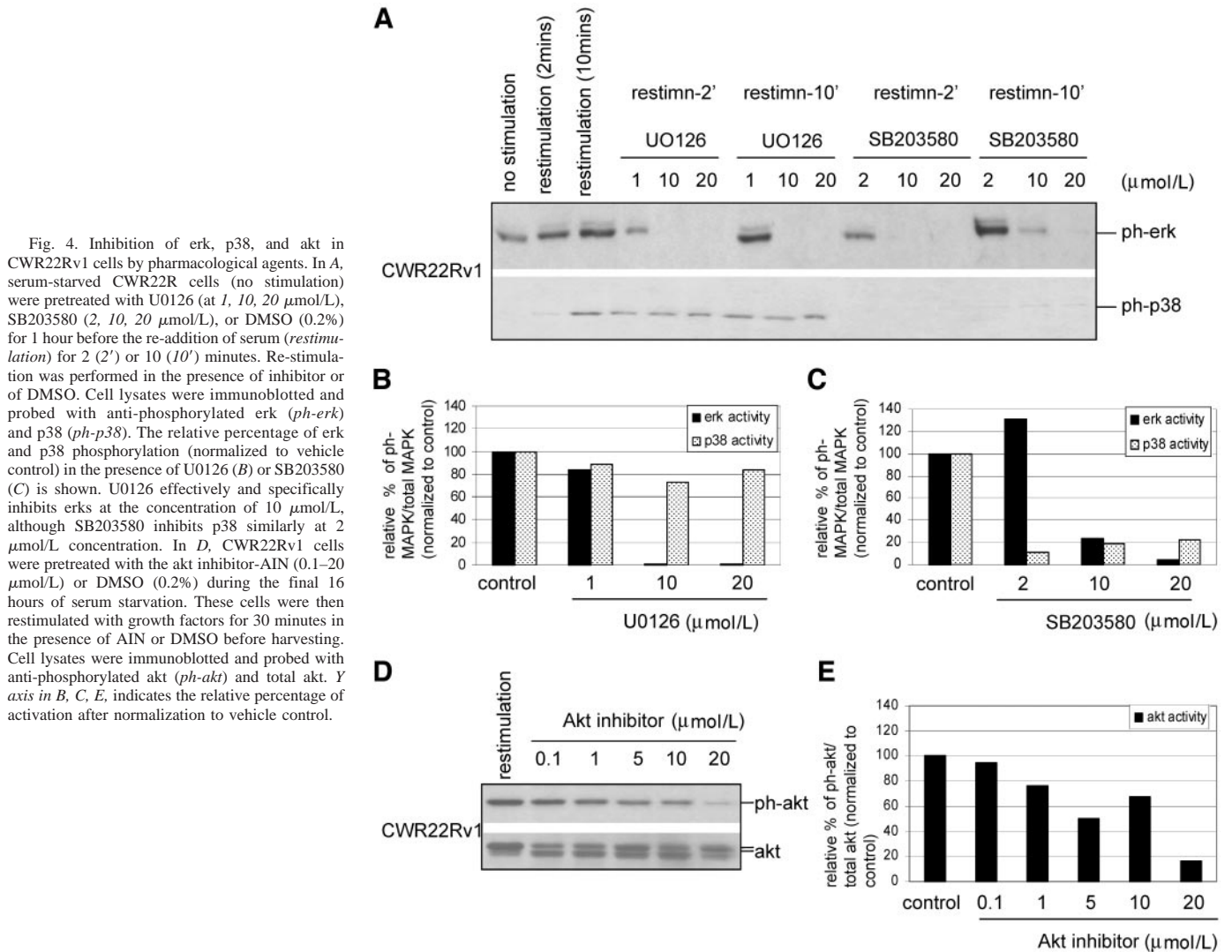


Fig. 4. Inhibition of erk, p38, and akt in CWR22Rv1 cells by pharmacological agents. In A, serum-starved CWR22R cells (no stimulation) were pretreated with UO126 (at 1, 10, 20 μmol/L), SB203580 (2, 10, 20 μmol/L), or DMSO (0.2%) for 1 hour before the re-addition of serum (restimulation) for 2 (2') or 10 (10') minutes. Re-stimulation was performed in the presence of inhibitor or of DMSO. Cell lysates were immunoblotted and probed with anti-phosphorylated erk (ph-erk) and p38 (ph-p38). The relative percentage of erk and p38 phosphorylation (normalized to vehicle control) in the presence of UO126 (B) or SB203580 (C) is shown. UO126 effectively and specifically inhibits erks at the concentration of 10 μmol/L, although SB203580 inhibits p38 similarly at 2 μmol/L concentration. In D, CWR22Rv1 cells were pretreated with the akt inhibitor-AIN (0.1–20 μmol/L) or DMSO (0.2%) during the final 16 hours of serum starvation. These cells were then restimulated with growth factors for 30 minutes in the presence of AIN or DMSO before harvesting. Cell lysates were immunoblotted and probed with anti-phosphorylated akt (ph-akt) and total akt. Y axis in B, C, E, indicates the relative percentage of activation after normalization to vehicle control.

stimulation). This compound was specific in that it does not affect p38 activation (Fig. 4A and B). SB203580 was highly effective in blocking p38 activation at concentrations of 2 to 20 μmol/L. It diminished erk activation at the higher concentrations of 10 to 20 μmol/L; however, as previously reported (28), limiting its use to 2 μmol/L for the specific inhibition of only p38 (Fig. 4A and C). At a concentration of 20 μmol/L, SP600125 inhibited Jnk (*i.e.*, jnk 1, 2, and 3) by >90% with less than a 20% inhibition of erk or p38, as previously reported (25). The phosphorylation of akt was effectively inhibited by 90% at 16 hours after exposure to a 20 μmol/L concentration of the akt inhibitor-AIN (Fig. 4D and E). On the basis of these results, UO126, SB203580, SP600125, and AIN were used at 10, 2, 20, and 20 μmol/L concentrations, respectively.

Activity of Erk and Jnk Is Required for Proliferation and Survival whereas P38 and Akt Activities Stimulate Only Proliferation of Normal Prostatic Epithelium. Exponentially growing PrECs were treated with UO126, SB203580, SP600125, and AIN, alone or in combinations (Fig. 5A). In the presence of erk inhibition via UO126 alone, or Jnk inhibition via SP600125 alone, the numbers of live cells were significantly ($P < 0.05$) reduced to levels below those normalized to the starting cell number, indicating that cell death occurred (Fig. 5A). This cytotoxic response was confirmed by time-lapse videomicroscopy. These studies documented that either erk or jnk inhibition alone caused cessation of cell motility and proliferation.

Morphologically, these two treatments induced different changes in cell shape. The inhibition of erk resulted in flattening of the cell (Fig. 5B versus C), whereas jnk inhibition resulted in cell shrinkage (Fig. 5B versus 5D). In both cases, however, these changes were associated with the death of the cells via cornification [*i.e.*, formation of SDS-insoluble cornified envelopes (ref. 29; Fig. 5E)]. This cornification phenotype was due to cross-linking of the cellular protein involucrin via up-regulation of transglutaminase during apoptotic cell death (30). Untreated normal PrECs expressed high levels of both involucrin and transglutaminase (Fig. 5F). The detection of both larger and smaller bands compared with the involucrin monomer (Fig. 5F) was consistent with a continuous cycle of transglutaminase-induced involucrin cross-linking and degradation, even in untreated normal prostatic epithelium. When these untreated PrECs were exposed to 1% SDS, the cells were completely solubilized, even though they contained detectable levels of cross-linked involucrin. In contrast, when PrECs were induced to die by either erk or jnk inhibition, such enhanced cross-linking resulted in cornified envelopes that were insoluble in 1% SDS treatment (Fig. 5E). Interestingly when PrECs were induced to undergo apoptotic death via an increase in the intracellular free calcium [induced by treatment with the SERCA (sarco/endoplasmic reticulum calcium ATPase) pump inhibitor thapsigargin, as described previously (31)], the cells also shrank, apoptosed, and underwent cross-linking, producing cornified envelopes

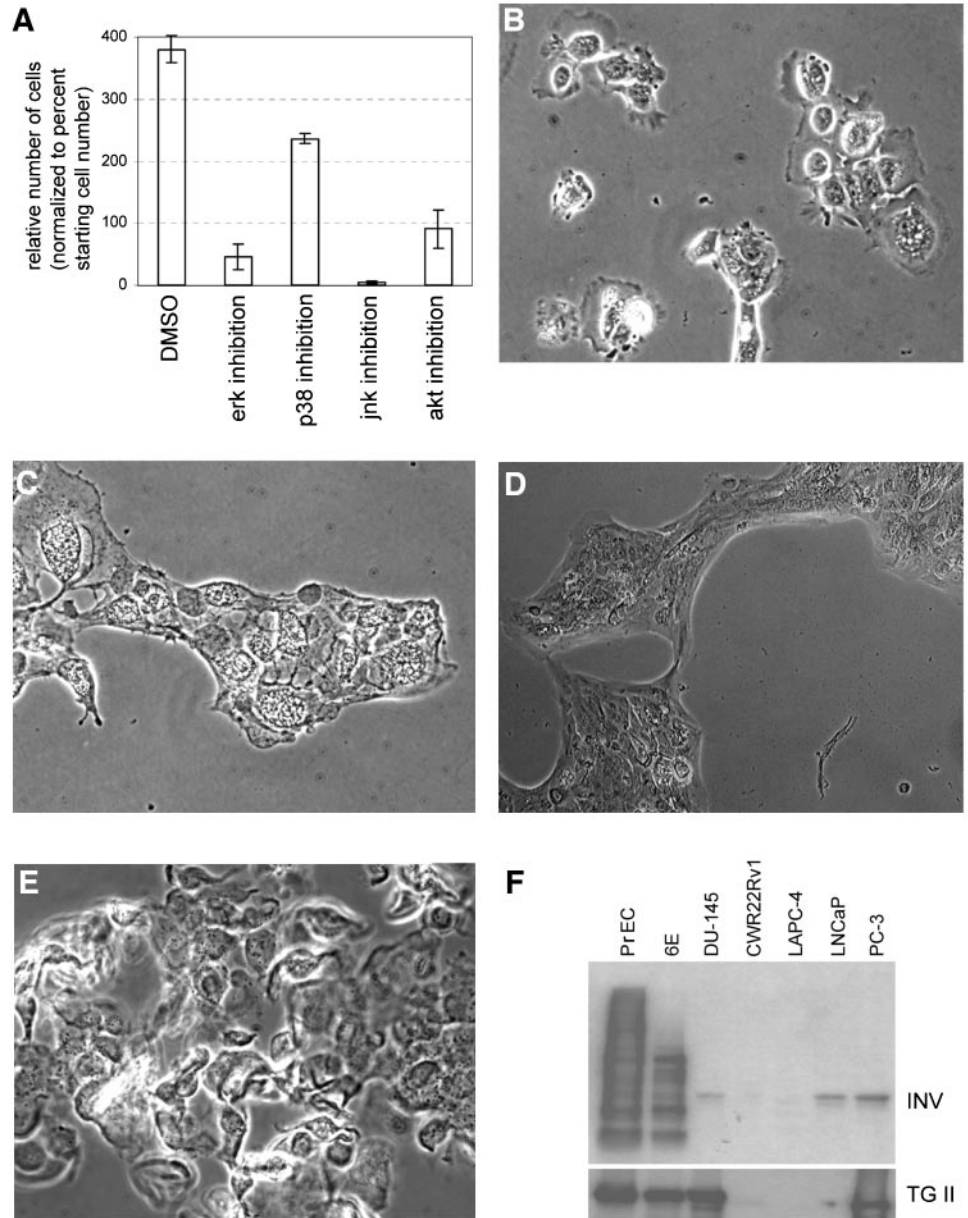


Fig. 5. Growth of normal prostate cells in the presence of MAPK and akt inhibitors. *A*, quantitation of live PrECs using MTT assays. PrECs were grown in complete medium containing 10 $\mu\text{mol/L}$ U0126 (*erk inhibition*), 2 $\mu\text{mol/L}$ SB203580 (*p38 inhibition*), 20 $\mu\text{mol/L}$ SP600125 (*jnk inhibition*), 10 $\mu\text{mol/L}$ AIN (*akt inhibition*), or 0.2% DMSO for a period of 7 days. Estimated percentage cell number was normalized to the starting cell number at day 0 (*i.e.*, 100%) and represented as mean \pm SEM of three independent experiments; $>100\%$, cell growth; $<100\%$, cell death. *B-E*, time-lapse videomicroscopy of PrECs over a period of 5 days. Cells were treated with 0.2% DMSO (*i.e.*, control; *B*), 10 $\mu\text{mol/L}$ U0126 (*C* and *E*), and 20 $\mu\text{mol/L}$ SP600125 (*D*). In *E*, SDS (0.1%) was added to U0126-treated cells. *F*, Western blot of lysates from normal (*PrEC*, *6E*) and malignant (*DU-145*, *CWR22Rv1*, *LAPC-4*, *LN-CaP*, *PC-3*) prostate epithelial cells, probed with antibodies against involucrin (*INV*) and transglutaminase II (*TG II*). *B*, *C*, *E*, $\times 200$; *D*, $\times 100$.

resistant to 1% SDS solubilization. These combined results demonstrated that formation of cornified envelopes is the characteristic end point for the apoptotic death of these normal transit-amplifying PrECs regardless of the inducing agent. Such death and cornification were not produced when PrECs were exposed to the p38 or akt inhibitors. Akt inhibition alone did result, however, in a complete suppression of net growth of PrECs (Fig. 5A). Using both time-lapse videomicroscopy and DAPI nuclear DNA staining, we confirmed that akt inhibition did not induce apoptotic death and cornification of these cells but, instead, prevented their proliferation (Fig. 5A). The inhibition of p38 alone had only a modest cytostatic effect (Fig. 5A).

Taken together, these results documented that simultaneous erk and jnk activity is both necessary and sufficient for transit-amplifying cell survival signaling. Unexpectedly, when erk and jnk are active, akt activity is not necessary for survival. Thus, there is either a redundancy between akt and the MAPK activities or else, akt is not required for survival signaling in TA cells. In contrast, although simultaneous erk and jnk activity may be necessary, they are not sufficient for

proliferation of TA cells. Such proliferation of TA cells requires additional signaling by akt.

Variation in the Requirement for MAPKs and Akt Activities in the Proliferation and Survival of Malignant Prostate Epithelial Cells. Exponentially growing malignant prostate cells were treated with erk, jnk, p38, and akt inhibitors alone or in combination (Figs. 6A–E). Whereas control and p38-inhibited CWR22Rv1 cells grew exponentially with an essentially equal rate, those treated alone with either the erk, jnk, or akt inhibitor grew at a reduced rate ($P < 0.05$; Fig. 6A). The growth reduction induced by combined treatment with all four inhibitors was significantly greater ($P < 0.05$) than that produced by any single inhibitor alone (Fig. 6A). Morphological and biochemical analyses demonstrated that none of the single or combination treatments induced cornification as assayed by 1% SDS insolubility. As a single agent, only jnk inhibition increased the extent of apoptosis as assayed by DAPI nuclear DNA staining (Fig. 6F). Thus, the growth retardation induced by erk and akt inhibition in CWR22Rv1 cells was due to a decrease in proliferation, not by

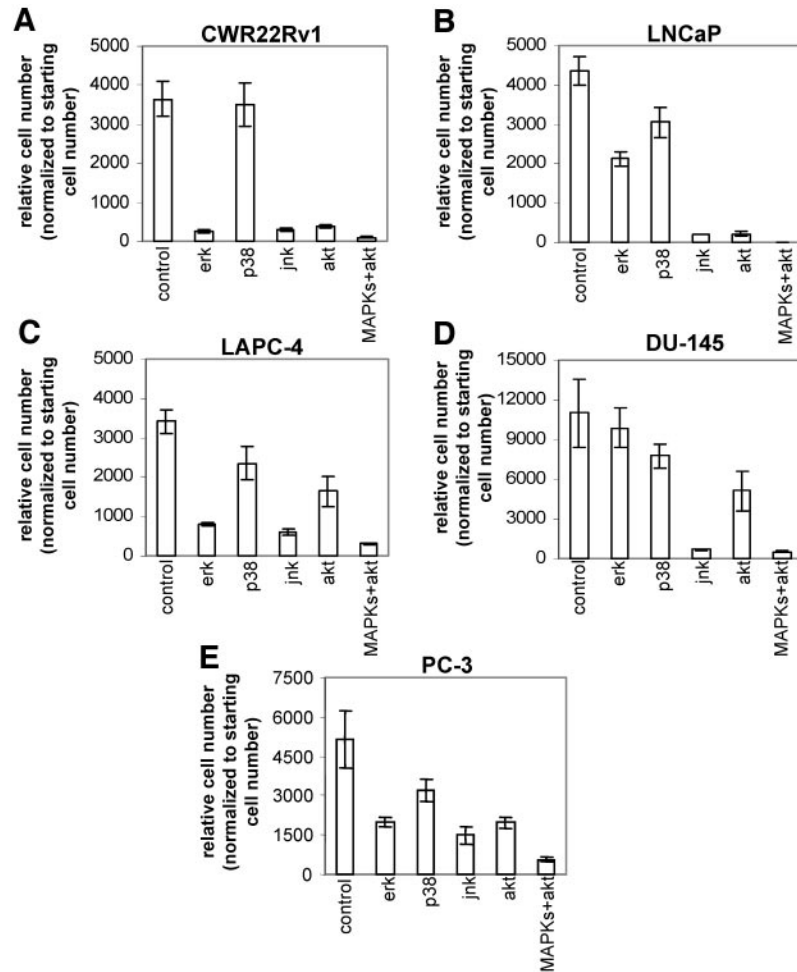


Fig. 6. Growth and apoptosis of malignant prostate epithelial cells in the presence of MAPK and akt inhibitors. Growth (A–E) and apoptosis (F) of malignant cells (*CWR22Rv1*, *LNCaP-LP*, *LAPC-4*, *DU-145*, *PC-3*) in the presence of 0.2% DMSO (*control*), erk inhibitor-10 $\mu\text{mol/L}$ U0126 (*erk*), p38 inhibitor-2 $\mu\text{mol/L}$ SB203580 (*p38*), jnk inhibitor-20 $\mu\text{mol/L}$ SP600125 (*jnkn*), akt inhibitor-10 $\mu\text{mol/L}$ AIN (*akt*), and combination of all above mentioned inhibitors (*MAPKs+akt*). In A–E, the number of viable cells was estimated by MTT assays at day 0 and day 5 (*DU-145*) or day 7 (*CWR22Rv1*, *LNCaP-LP*, *LAPC-4*, *PC-3*). The number of cells estimated after 5 or 7 days was normalized to the starting cell number (*i.e.*, 100%). The percentage cell number is represented as the means \pm SEM of three independent experiments. In F, the percentage apoptosis is represented as the means \pm SEM of three independent experiments.

F Percentage apoptosis after 5 day's exposure to indicated treatment						
Cell Line	Control	erk inhibition	jnkn inhibition	p38 inhibition	akt inhibition	erk+jnkn+p38+akt inhibition
CWR22v1	9 \pm 2	15 \pm 3	21 \pm 4*	8 \pm 7	13 \pm 4	75 \pm 11*
LNCaP-LP	11 \pm 4	10 \pm 5	43 \pm 7*	10 \pm 6	42 \pm 3*	89 \pm 9*
LAPC-4	12 \pm 5	14 \pm 5	13 \pm 6	10 \pm 4	12 \pm 3	50 \pm 9*
DU-145	15 \pm 6	17 \pm 3	45 \pm 9*	12 \pm 6	19 \pm 3	90 \pm 4*
PC-3	13 \pm 4	15 \pm 7	17 \pm 4	11 \pm 7	16 \pm 2	39 \pm 6*

* $p > 0.05$ compared with value for control DMSO-treated group

induction of cell death (*i.e.*, cytostatic effect). In contrast, jnk inhibition alone not only induced a cytostatic effect but enhanced the percentage of cells undergoing apoptosis. This resulting cytotoxic enhancement was synergized by the inhibition of akt and the other MAPKs (Fig. 6F).

When LNCaP-LP cells were treated with erk, jnk, p38, or akt inhibitors, growth was inhibited ($P < 0.05$; Fig. 6B). For the erk and p38 inhibitors, this growth inhibition was not associated with an increase in apoptosis as assayed by DAPI staining (Fig. 6F). In contrast, jnk or akt inhibition alone resulted in an increase ($P < 0.05$) in apoptosis (Fig. 6F), demonstrating that both jnk and akt activity are critical for survival of LNCaP cells. In contrast, LAPC-4 growth was not affected by p38 inhibition but was growth inhibited (*i.e.*, 2–3-fold

reduction) by erk, jnk, or akt inhibitors alone ($P < 0.05$), compared with controls (Fig. 6C). This latter effect was due to decreased proliferation and not to enhanced apoptosis (Fig. 6F). The best growth inhibition for LAPC-4 cells was produced by combining all four inhibitors (Fig. 6C). Only this combinational approach was able to induce an enhanced level of apoptosis (Fig. 6F). The growth of DU145 cells was not significantly inhibited by either erk or p38 blockade (Fig. 6D and F). Whereas akt inhibition did inhibit the proliferation of DU145 cells, it did not enhance apoptosis of these cells (Fig. 6F). Inhibition of jnk alone did decrease the growth of these cells (Fig. 6D), via its ability to increase apoptosis (Fig. 6F). This apoptotic response was further enhanced by combination with the other inhibitors (Fig. 6D and F). The growth of PC-3 cells was

decreased by each of the inhibitors (Fig. 6E). This was due to a cytostatic effect with apoptosis of PC-3 cells being enhanced only by the combination of all four inhibitors (Fig. 6F). In conclusion, monoinhibition of erk, jnk, or akt produced a reduction in cell number in a majority of the malignant cell lines. However, only jnk inhibition produced an increase in apoptosis. Combinatorial inhibition of erk, p38, jnk, and akt resulted in an enhanced induction of apoptosis and a reduction of growth as compared with the effect of inhibition of any single kinase. Thus, these results demonstrate that erk, p38, jnk, and akt have redundant roles in the regulation of survival of prostate cancer cells.

DISCUSSION

The present studies have documented that the survival of normal prostatic TA cells requires simultaneous signaling by erk and jnk via nonredundant downstream effectors. Unexpectedly, akt signaling is not required for survival even though its activity is required for the proliferation of these normal TA cells. In contrast, p38 signaling only enhances but is not critically required for proliferation and has no effect on the survival of the TA cells. These studies have also documented that during progression of prostatic cancer to the lethal phenotype of androgen independence, there are significant changes in the regulatory signaling network controlling survival and proliferation. These changes result in an enhanced redundancy in the downstream signaling pathway that maintains the survival of these malignant cells. No longer does erk inhibition alone result in cell death, and even the inhibition of jnk by itself produces only a limited apoptotic response. Also, inhibition of akt induces apoptosis of only one (*i.e.*, LNCaP-LP) of the five malignant lines tested. Induction of a strong and generalized apoptotic response in all five of the androgen-independent prostate cancer cell lines requires the combinatorial inhibition of akt, erk, p38, and jnk. These results are consistent with an acquired ability by malignant prostate cells for akt and the MAPKs redundantly to activate the downstream target effectors of survival that are normally activated only by erk and jnk.

What is the mechanism for such enhanced downstream redundancy in survival signaling in malignant *versus* normal prostate cancer cells? There is a growing body of evidence that androgen receptor in either a ligand-dependent or -independent manner can “cross-talk” with other signaling pathways in prostate cancer cells (6). Because normal prostatic TA cells do not express androgen receptor, such “cross-talk” is not possible with the lack of redundancy for the erk and jnk survival signaling in these cells. Such an androgen receptor-dependent explanation is also not consistent, however, with the fact that PC-3 and DU145 prostate cancer cells also do not express androgen receptor but still have enhanced redundancy in survival signaling. An alternative possibility is that during cancer progression, molecular changes occur stochastically because of the inherent genetic instability of prostate cancer cells driven by chronic and/or acute inflammation (32). In this inflammatory microenvironment, one of the first and most consistent molecular changes is the methylation of the promoter of the detoxifying enzyme glutathione *S*-transferase π isoform (*i.e.*, GST π) resulting in a decrease in its expression in prostate cancer (32). Such a characteristic decrease in GST π expression results in a significant growth advantage (33) because, now, GST π cannot bind to and inhibit jnk activity (34). Thus, increased jnk activation via a decrease in GST π provides a driving force for the genetic instability of prostate cancer. Any genetic change that enhances the redundancy in downstream effectors of survival pathways would provide a selective growth advantage. Such a process of genetic instability coupled with clonal selection has been documented during progression of prostatic cancer in animal model systems (35) and could explain why such

enhanced redundancy in survival pathways is consistently observed during the progression to an androgen-refractory state.

In conclusion, because of the enhanced redundancy in downstream effectors for survival pathways, only the combined inhibition of akt and MAPK signaling induces sufficient apoptosis to produce a therapeutic effective response in all of the androgen-independent prostate cancer cell lines tested. Such redundancy is not unlimited, however (36, 37), and provides the critical rationale for developing combinatorial approaches to signal transduction inhibition. Such combinatorial strategies are currently being tested *in vivo* in nude mice orthotopically bearing the same panel of androgen-independent human prostate cancers characterized in the present studies.

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