Implication of Cell Kinetic Changes during the Progression of Human Prostatic Cancer[1]

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Abstract

The daily percentage of cells proliferating and dying were determined for normal, premalignant,
and cancerous prostatic cells within the prostate as well as for prostatic cancer cells in lymph
node, sort tissue, and bone metastases from untreated and hormonally failing patients. These data
demonstrate that normal prostatic glandular cells have an extremely low but balanced rate of cell
proliferation and death (*i.e.,* both <0.20%/day). This results in a steady-state, self-renewing
condition in which there is no net growth, although the glandular cells are continuously being
replaced (*i.e.,* turnover) every 500 ± 79 days. Transformation of these cells into high-grade
prostatic intraepithelial neoplastic cells initially involves an unbalanced increase in the daily
percentage of cells proliferating *versus* dying, such that net continuous growth occurs (*i.e.,* mean
doubling time, 154 ± 22 days). As these early proliferation lesions continue to grow into late stage
high-grade prostatic intraepithelial neo-plastic cells, the daily percentage of cells dying increases
further to a point equaling the daily percentage of proliferation. This results in cessation of net
growth while inducing a 6-fold increase in the turnover time of these cells (*i.e.,* 56 ± 12 days),
increasing their risk of further genetic changes. The transition of late stage high-grade prostatic
intraepithelial neoplastic cells into localized prostatic cancer cells involves no further increase in
proliferation but a decrease in death resulting in net continuous growth of localized prostatic
cancers with a mean doubling time of 475 days. As compared to localized prostatic cancer cells,
metastatic prostatic cancer cells within lymph nodes or bones of untreated patients have an
increase in daily rate of proliferation coupled with a reduction in their daily percentage of cell
death, producing net growth rates with a mean doubling time of 33 ± 4 days and 54 ± 5 days,
respectively. Remarkably, there is no further increase in proliferation in hormonally failing
patients, but instead an increase in the daily percentage of androgen-independent prostatic cancer
cells dying within sort tissue or bone metastases. These changes result in doubling times which are
two to three times longer (*i.e.,* 126 ± 21 and 94 ± 15 days) in these lymph node and bone meta-
static sites, respectively, compared to similar sites in hormonally untreated patients. These data

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demonstrate that the daily percentage of proliferation for either androgen-dependent or -independent metastatic prostatic cancer cells is remarkably low (*i.e.,* <3.0%/day), consistent with why antiproliferative chemotherapy has been of such limited value against such metastatic cells. These results also suggest that prostatic carcinogenesis starts in the second to third decade of life and may require over 50 years for progression to pathologically detectable metastatic disease.

Introduction

Metastatic prostatic cancers, like the normal prostates from which they arise, are sensitive to androgenic stimulation of their growth. This is due to the presence of androgen-dependent prostatic cancer cells within such metastatic patients. These cells are androgen dependent since androgen stimulates their daily percentage of proliferation (*i.e.,* K_p) while inhibiting their daily percentage of death (*i.e.,* K_d; Ref. 1). In the presence of adequate androgen, continuous net growth of these dependent cells occurs since the daily percentage of cells proliferating exceeds the daily percentage of cells dying. In contrast, following androgen ablation, androgen-dependent prostatic cancer cells stop proliferating and activate a cellular suicide pathway, termed programmed cell death or apoptosis (2). This activation results in the elimination of these androgen-dependent prostatic cancer cells since their daily percentage of cell death now exceeds their daily rate of cell of proliferation. Due to this elimination, 80–90% of all men with metastatic prostatic cancer treated with androgen ablation therapy have an initial positive response (3). All of these patients eventually relapse to a state unresponsive to further antiandrogen therapy, no matter how completely given (3). This is due to the heterogeneous presence of androgen-independent prostatic cancer cells within such metastatic patients (4). These latter cells are androgen-independent since their rate of proliferation exceeds their rate of cell death even after complete androgen blockage is performed (5).

Attempts to use nonandrogen ablative chemotherapeutic agents to adjust the kinetic parameters of these androgen-independent prostatic cancer cells so that their daily percentage of death exceeds their daily percentage of proliferation have been remarkable in their lack of success (6). The agents tested in androgen ablation-failing patients have been targeted at inducing DNA damage directly or indirectly via inhibition of DNA metabolism or repair (6). These agents are thus critically dependent upon an adequate rate of proliferation to be cytotoxic (7). *In vitro* cell culture studies have demonstrated that when androgen-independent, metastatic, prostatic cancer cells have a high daily percentage of cell proliferation (*i.e.,* K_p 50%), these cells are highly sensitive to the induction of programmed cell death via exposure to the same antiproliferative chemotherapeutic agents which are of limited value when used *in vivo* in prostatic cancer patients (8). The apparent paradox between the *in vitro* and *in vivo* responsiveness to the same chemotherapeutic agents by androgen-independent prostatic cancer cells may reflect major differences in the daily percentage of proliferation occurring in the two states. Likewise, for chemotherapeutic agents to be effective, not only must the cancer cells have a critical daily percentage of proliferation but also a critical sensitivity to induction of cell death (9). This sensitivity to induction of cell death is reflected in the magnitude of the daily percentage of cell death in the untreated condition (9).

Thus, knowledge concerning the magnitude of the daily percentage of cell proliferation (*i.e.*, K_p) and death (*i.e.*, K_d) for androgen-independent metastatic prostatic cancer cells could provide a logical explanation of why the previously tested chemotherapeutic agents have been of limited success against these devastating cells. PSA [4] is a unique prostatic marker whose serum level is related to prostatic cancer burden (10, 11). Several studies have attempted to use serial measurements of PSA levels to estimate the growth rate of prostatic cancer cells in patients with both localized or metastatic disease. Using serial serum PSA values, doubling times for localized *versus* metastatic disease were reported to be 2.4 ± 0.6 years *versus* 1.8 ± 0.2 years in the Carteret *al.* (10) study and 5.8 *versus* 3.6 years in the Schmid *et al.* (11) study. Since it requires at least 30 population doublings for a tumor to reach 1 cm^3 (12), these PSA-based growth rates predict that it should take minimally 72 years, based on the Carteret *et al.* (10) data, or 174 years, based on the Schmid *et al.* (11) data, for a patient to develop a 1 cm^3 primary prostatic cancer. Since the average time of diagnosis for clinical prostatic cancer is 72 years of age (13), these PSA-based calculations clearly are underestimates of the true growth rates for prostatic cancer cells. To obtain more accurate estimates of the growth rates of prostatic cancers and to clarify how these cell kinetic parameters are affected during prostatic cancer progression, the present study was performed. In this study, the *in vivo* daily percentage of cell proliferation and death were determined for normal, premalignant, and cancerous prostatic cells within the prostate as well as for prostatic cancer cells in lymph node, soft tissue, and bone metastases from untreated and hormonally failing patients.

Materials and Methods

Tissue

For determination of cell kinetic parameters of normal prostate glandular cells, prostatic intraepithelial neoplastic cells, and primary prostatic cancer cells, radical prostatectomy specimens were used. Prostates from 27 patients undergoing radical prostatectomy for pathological stage B localized prostatic cancer were processed for routine formalin fixation, paraffin embedding, and histological sectioning. Consecutive 4-μm-thick step sections were obtained for each prostate with representative sections being hematoxylin and eosin stained to determine the total cancer volume (*i.e.*, 2.9 ± 0.3 cm^3; range, 0.3–7.5 cm^3) and Gleason sum (*i.e.*, range, 4–10) and to define the area of prostatic cancer and the areas of peripheral prostatic tissue composed of only normal prostatic glandular cells. In this way, localized prostatic cancer cells could be age and patient matched to normal prostatic glandular cells for the kinetic analysis. An additional group of 20 prostates, also obtained from patients undergoing radical prostatectomy, was used to obtain histological sections containing high-grade PIN not associated with cancer (*i.e.*, early stage PIN) from 10 patients and sections containing high-grade PIN adjacent to primary prostatic cancer (*i.e.*, late stage PIN) from 10 patients. Classification of these lesions as high-grade PIN was as described previously (14). Pelvic lymph nodes containing metastatic prostatic cancer cells from a series of 30 patients with pathological stage D$_1$ metastatic prostatic cancer were also used. Also used were 23

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[4]The abbreviations used are: PSA, prostatic specific antigen; PIN, prostatic intraepithelial neoplasia; GF, growth fraction; Tc, intermitotic cell cycle time; BBRF, Biological Research Faculty and Facilities; TTF, terminal transferase fraction; TT, terminal transferase.

bone metastases and 5 soft tissue metastases (*i.e.*, from epidural, bronchus, breast, laminectomy, and mesenteric/omental sites) obtained from a series of patients with pathological D_2 metastatic prostatic cancer whose treatment status and response data were known.

Determination of the Daily Rate of Cell Proliferation (K_p)

The K_p value, expressed as the percentage of a particular cell type proliferating per day, was calculated by dividing the GF for the particular cell type by the intermitotic Tc, expressed in days, and then multiplying this number by 100 (15). The GF for a particular cell type was determined by immunocytochemical staining of histological sections using the commercially available mouse mAb MIB1 (AMAC, Westbrook, Maine) to detect cells in the cell cycle. This mAb has been demonstrated by Cattoretti *et al.* (16) to recognize epitopes in the Ki67 antigen in microwave-processed formalin-fixed, decalcified, paraffin sections. The Ki67 antigen is a nuclear nonhistone protein of 395 and 345 kDa present in all parts of the proliferative cell cycle (*i.e.*, G_1, S, G_2, and mitosis) but is absent when cells are out of cycle (*i.e.*, in G_0; Ref. 16). For these immunocytochemical stains, the microwave protocol described by Cattoretti *et al.* (16) was used, except that the secondary antibody used was a biotinylated rabbit anti-mouse lgG obtained from Vector (Burlington, CA) and detection was via a Vectostain ABC peroxidase kit (Vector) using diaminobenzamidine/nickel as the peroxidase substrate. After immunocytochemical staining, sections were counterstained with ethyl green. Two thousand cells per cell type per patient were chosen using the random sampling technique as we have described previously (17), and the fraction of cells whose nuclei were positively stained with the M1B1 antibody determined to calculate the GF.

The T_c was determined using primary cultures of both normal prostatic glandular cells and prostatic cancer cells. To do this normal prostatic glandular and cancerous tissues were obtained from radical prostatectomy specimens from patients with clinically localized prostatic cancers. These tissues were enzymatically dissociated into epithelial organoids by 8-12-h treatment with a 0.05% collagenase (GIBCO), 0.75% BSA (GIBCO), and antibiotics in RPMI 1640 media. The resultant epithelial organoids were plated in tissue culture dishes coated with a commercially available attachment solution containing bovine fibronectin, collagen, and BSA [*i.e.*, FNC coating mix (cat. AF-10) from BRFF, Ijamsville, MD]. The cells were maintained using a commercially available serum-free tissue culture media containing 10^{-8} M dihydrotestosterone (*i.e.*, HPC-1) from BRFF supplemented with 25 ng/ml cholera toxin, 5 ng/ml epidermal growth factor, 5 μg/ml insulin, and 100 μg/ml bovine pituitary extract (additive commercially available from BRFF). In this media, prostatic epithelial cells, but not the fibroblasts, attach and spread over a 4-day period. These primary epithelial cultures were time-lapse video recorded over the next week using an inverted microscope as described previously (18). Using these video recordings, the time between successive mitosis (*i.e.*, T_c) for both normal or malignant prostatic cells from five distinct patients were determined to be 48 ± 5 h (*i.e.*, 2 days). To determine whether the T_c changes when prostatic cancer progresses from a localized to a more advanced malignant phenotype, the DU-145, PC-3, and LNCaP human prostatic cell lines (*i.e.*, each established from a metastatic site) were video recorded as described previously (18). The mean T_c value was determined to be 48 ± 6 h for these three cell lines. These results demonstrated that there is

no change in the T_c value during the progression from localized to more advance prostatic cancer. Thus, a value of 2 days was used as the T_c value for normal and malignant prostatic cells.

Determination of the Daily Rate of Cell Death (K_d

The K_d value, expressed as the percentage of a particular cell type dying per day, was calculated by dividing the fraction of the cells whose DNA is end labelable with exogenous *in situ* treatment with purified terminal transferase (*i.e.*, termed TTF) by the half-life of the labeled cells (*i.e.*, termed $TT_{1/2}$) and then multiplying this number by 100. This calculation is based upon the fact that within the prostate, cell death normally occurs via programmed (*i.e.*, apoptotic) cell death and that during this programmed death, genomic DNA is double-strand fragmented, producing large numbers of free 3′-hydroxyl deoxynucleotide ends (19, 20). These newly produced 3′-hydroxyl groups can be end labeled *in situ* in formalin-fixed, paraffin-embedded histological sections as described by Gavrieli *et al.* (21) by incubation of the deparafinated sections with 300 units/ml purified terminal deoxynucleotide transferase (*i.e.*, TT; Boehringer Mannheim) and 6.25 μM biotinylated dUTP (Boehringer Mannheim) with subsequent detection of the end-labeled DNA performed with Vectostain ABC peroxidase kit using diaminobenzamidine/nickel as substrate. Sections were counterstained with ethyl green. Two thousand cells per cell type per patient were chosen using the random sampling technique described previously (17), and the fraction of cells whose nuclear DNA was positively end labeled but whose nuclei had not yet undergone apoptotic fragmentation was determined to calculate the TTF. Cells whose nuclear DNA was positively end labeled, but whose nuclei had already undergone apoptotic fragmentation, were excluded from the TTF determination. This exclusion is based on the observation that there can be substantial variation between the length of time such apoptotic cell fragments remain before becoming undetectable due to the phagocytosis and degradation by neighboring cells in normal and malignant tissue. In contrast to the variation in the time apoptotic cell fragments remain detectable, 12 h is consistently the average half-life (*i.e.*, $TT_{1/2}$) for cell nuclei to be terminal transferase end labelable before undergoing apoptotic fragmentation. This mean $TT_{1/2}$ value was determined by treating primary cultures of both normal and prostatic cancer cells from five separate patients and three established cell lines (*i.e.*, DU-145, PC-3, and LNCaP) with the 10 μM of the calcium ionophore, ionomycin (Calbiochemical, CA), for 4 days *in vitro.* As described previously, such chronic treatment with ionomycin elevates the intracellular free Ca^{2+} (Ca_i) and within 4 days induces programmed death of prostatic cells (18). After 4 days, triplicate cultures were fixed with 10% formalin, terminal transferase end labeled as described, and the TTF determined. Additional cultures had their ionomycin-containing media replaced with media lacking ionomycin, and then at 6-h intervals triplicate cultures were fixed and TTF determined. Previously we have demonstrated that once ionomycin is removed from the media, the Ca_i returns to baseline and no additional cells subsequently initiate their programmed death pathway (18). Thus, by plotting the log of TTF *versus* time of removal of ionomycin, the half-life of terminal transferase-labeled nuclei before they undergo apoptosis (*i.e.*, $TT_{1/2}$), was determined to be 12 ± 2 h (*i.e.*, 0.5 days) for normal prostatic cells, 12 ± 3 h for the localized prostatic cancer cells, and 12 ± 3 h for the cell lines. Thus, a value of 0.5 days was used as the $TT_{1/2}$ for normal and malignant prostatic cells.

Determination of Net Growth Rate, Doubling Time, and Turnover Time

The net growth rate, expressed as the percentage of cells accumulating per day, was determined by subtracting the K_d value from the K_p value for the particular cell type (15). When $K_p > K_d$, the doubling time was calculated according to the formula: $\ln2/[K_p - K_d]$ (15). When $K_p = K_d$, the turnover time (TT) was determined using the formula: $T_T = 1/K_p$ (15).

Statistical Analysis

Numerical values are expressed as the mean ± SE. Statistical analyses of significance were made by a one-way ANOVA with the Kruskal-Wallis test.

Results

Kinetic Parameters for Normal and Premalignant Prostatic Glandular Cells

The percentage of normal glandular cells proliferating per day (*i.e.*, K_p) is remarkably low in the prostate, Table 1 and Fig. 1*A*. However, this low rate of glandular cell proliferation is large enough to balance the equally low percentage of these cells spontaneously dying per day (*i.e.*, K_d, Table 1). This demonstrates that these normal cells are in a steady-state, self-renewing condition in which neither over-growth nor regression of these cells continuously occurs. During this maintenance condition, the turnover time (*i.e.*, the time required to renew these cells) is 500 ± 79 days.

When such normal prostatic glandular cells undergo transformation into early stage high-grade PIN, there is a 6.9-fold increase ($P < 0.05$) in the K_p value for these cells (Fig. 1*C*), with only a 4-fold increase ($P < 0.05$) in the K_d value (Table 1 and Fig. 1*8*). This leads to a predicted net growth rate (*i.e.*, K_p-K_d) of 0.45 ± 0.11% of cells accumulating per day. This translates into a doubling time of 154 ± 22 days for these early high-grade PINs. For late stage high-grade PIN, there is a 9-10-fold increase ($P < 0.05$) in both the K_p and K_d values as compared to the normal prostatic glandular cells. Since the $K_p = K_d$, however, late stage high-grade PINs do not continue to undergo net growth but instead are in a steady state of self-renewal. These late stage high-grade PINs, however, do have a 9-fold higher ($P > 0.05$) turnover time (*i.e.*, 56 ± 12 days for late stage high-grade PIN *versus* 500 ± 79 days for normal prostatic glandular cells), thus increasing their risk of further genetic changes needed to acquire the ability for net continuous growth.

Kinetic Parameters for Localized Prostatic Cancer Cells

There is an ≈6-fold increase ($P < 0.05$) in K_p values for localized prostatic cancer cells within the prostate of pathological stage B patients compared to normal prostatic glandular cells (Table 1 and Fig. 1*E*). However, there is no difference between the K_p values for high-grade PIN and localized prostatic cancer (Table 1). Likewise, there is no correlation between K_p values and patient age or primary tumor volume and the histological tumor grade (*i.e.*, Gleason sum; Note: there were no primary cancers with a Gleason sum below 4 in this series). As compared to the high K_d value in late stage high-grade PIN, there is a 40% decrease ($P < 0.05$) in the K_d values for the localized prostatic cancer cells which is still, however, 6–7-fold higher than for the normal prostatic glandular cells (Table 1 and Fig. 1*D*).

There is no correlation between the K_d values and patient age or primary tumor volume histological tumor grade. These kinetic changes predict a net growth for these prostatic cancer cells. The mean net growth rate for low *versus* high Gleason sum localized prostatic cancers is 0.12 ± 0.03 *versus* $0.14 \pm 0.04\%$ of cells accumulating/day, respectively. This translates into a mean doubling time of 577 ± 68 and 495 ± 56 days, respectively, for low *versus* high Gleason sum localized cancers.

Kinetic Parameters for Metastatic Prostatic Cancer Cells in Metastatic Sites

The mean K_p value of metastatic prostatic cancer cells in pelvic lymph nodes of hormonally untreated pathological stage D_1 patients was increased by 15-fold ($P > 0.05$) compared to the normal prostate glandular cells and 1.5–2-fold ($P > 0.05$) compared to either late stage prostatic intraepithelial neoplastic cells or localized prostatic cancer cells. There was no correlation between the K_p values and patient age or Gleason sum. (Note: there were no metastases with a Gleason sum below 6.) Likewise, there is no correlation between the K_d values and patient age or Gleason sum in the metastatic prostatic cancer cells growing in pelvic lymph nodes of untreated patients (Table 1). The K_d values for these metastatic cells in lymph nodes were reduced $\approx 40\%$ ($P < 0.05$) and $\approx 60\%$ ($P < 0.05$) compared to localized prostatic cancer cells and late stage high-grade prostatic intraepithelial neoplastic cells, respectively. These kinetic changes result in a predicted net growth rate of $2.1 \pm 0.30\%$ of prostatic cancer cells accumulating in lymph nodes/day. This translates into a doubling time of 33 ± 4 days.

The mean K_p value for metastatic prostatic cancer cells within the bone of untreated patients was 36% higher ($P < 0.05$) than that of localized prostatic cancer cells and 10.7-fold increased ($P > 0.05$) compared to normal prostatic glandular cells (Table 1). There is also an approximately 50% reduction ($P < 0.05$) in the K_d value in these bone metastases as compared to localized prostatic cancer cells and a 60% reduction ($P < 0.05$) compared to late stage high-grade prostatic intraepithelial neoplastic cells (Table 1 and Fig. I*F*). The K_d value for bone, however, is still 3.8-fold ($P > 0.05$) higher than for normal prostatic glandular cells. Thus, the predicted net growth rate for these bone metastatic prostate cancer cells is $1.28 \pm 0.23\%$ of prostatic cancer cells accumulating in the bone/day, which translates into a doubling time of 54 ± 5 days. This value is nearly 8–9-fold faster than that of localized prostatic cancer cells but approximately 40-fold lower ($P < 0.05$) than those for the net growth rate of metastatic cells in lymph nodes.

In hormonally untreated patients, it is unknown what proportion of the metastatic prostatic cancer cells analyzed for the K_p and K_d determinations are androgen dependent *versus* independent. In contrast, in patients failing androgen ablation therapy, these metastatic cancer cells are androgen independent. Androgen-independent metastatic prostatic cancer cells in either bone or soft tissue sites in patients failing hormonal treatment have no significant difference in their K_p values compared to metastatic cells in the respective sites in hormonally untreated patients. There is, however, a 2-fold increase in the K_d values ($P < 0.05$) for androgen-independent metastatic prostatic cancer cells in soft tissue and bone sites in failing *versus* hormonally untreated patients (Table 1). These kinetic changes result in predicted net growth rates of $0.55 \pm 0.09\%$ and $0.74 \pm 0.12\%$ of the androgen-independent

metastatic prostatic cancer cells accumulating/day respectively in soft tissue *versus* bone sites. This translates into a doubling time of 126 ± 21 days and 94 ± 15 days respectively for soft tissue *versus* bone sites in these hormonally failing patients.

Discussion

The daily percentage of cells proliferating (*i.e.*, K_p) can be calculated from the formula: $K_p =$ growth fraction/intermitotic cell cycle time (days) $\times 100$ (15). The GF can be determined using immunocytochemical staining with the M1B1 mAb to detect the fraction of a particular cell type positively expressing the Ki67 antigen in formalin-fixed, paraffin-embedded, histo-logical sections (16). The Ki67 antigen is a nuclear nonhistone protein expressed in all parts of the proliferative cell cycle, but absent where cells are out of cycle (16, 22). Baisch and Gerdes (23) demonstrated that identical GF values were obtained using Ki67 immunostaining or other methods, including stathmokinetic measurements using colchicine blockade or flow cytometry measurement with bromodeoxyuridine labeling, except when cells are nutritionally deprived. When cells are nutritionally deprived, the GF determined on the basis of Ki67 immunostaining is higher than the GF determined by S-phase progression (23). This suggests that under such nutritional deprivation, cells spend longer times in G_1 than normal (*i.e.*, T_c increase). *In vivo,* the hallmark of such nutritional deprivation is the appearance of morphological signs of necrosis (*i.e.*, areas within tissues in which multiple adjacent cells have undergone cellular edema, vacuolization, and/or cellular lysis). Based on such standard morphological criteria, necrosis is not a commonly identified morphological features in any of the tissue samples examined in this study. Thus, within these samples, GF estimated on the basis of positive staining for the Ki67 antigen should be reasonably accurate and allow valid estimation of the K_p values, if appropriate T_c values are used in the calculation.

Presently, it is impossible to determine the T_c value within a particular cancer specimen based on immunocytochemical staining of a formalin-fixed, paraffin-embedded histological section without injecting the patient with nucleotide precursors and harvesting and analyzing repeated biopsies after varying times (9, 15). Thus, in order to allow retrospective analysis of archival pathology specimens from untreated patients a "standardized" value for T_c must be estimated and used in the K_p calculation. In the present studies, we used time-lapse videomicroscopy to determine the T_c directly for: (*a*) normal prostatic glandular cells, (*b*) primary cultures of pathological stage B (*i.e.*, localized) prostate cancer cells, and (*c*) continuously passageable cell lines established using prostatic cancer tissue obtained from metastatic sites from patients failing hormonal therapy. Thus, within this series of samples, the full range of progression (*i.e.*, normal prostate $\rightarrow$ localized prostatic cancer $\rightarrow$ metastatic androgen-independent prostatic cancer) is represented. Regardless of the progressional state, the mean T_c was determined to be 2 days within this series of samples. A similar mean T_c value of 2 days has been reported by Tubiana and Malaise (9) using the percentage of [^{3}H]thymidine-labeled mitosis method on a series of more than 40 human solid cancers (9). Thus, T_c value of 2 days was used in all of our calculations. The accuracy of these calculated K_p values, therefore, is related to the validity of the estimated 2 days for the T_c value.

For determination of the daily percentage of prostatic cells dying (*i.e.*, K_d), cell death was assumed to occur exclusively via apoptosis and not via necrosis in the various prostatic tissue samples analyzed. This assumption is based on histological evaluation using standard morphological criteria which demonstrated that necrosis is not a commonly identified morphological feature in any of the tissue samples examined. Since DNA fragmentation is a characteristic of apoptotic death, terminal transferase end labeling can be used to identify cells undergoing apoptosis in histological sections (21). Thus, the daily percentage of cells dying was determined using the formula: K_d = fraction of cells terminal transferase labelable but not yet undergoing fragmentation into apoptotic bodies (TTF) divided by the half-life (in days) of terminal transferase end-labeled nuclei before undergoing fragmentation into apoptotic bodies ($TT_{1/2}$) × 100. Presently, it is impossible to determine the $TT_{1/2}$ value for each individual cell type based on immunocytochemical staining alone. Therefore, the average half-life for terminal transferase end-labeled cell nuclei before undergoing fragmentation into apoptotic bodies ($TT_{1/2}$) was determined for: (*a*) normal prostatic glandular cells, (*b*) primary culture of pathological stage B (*i.e.*, localized) prostatic cancer cells, and (*c*) continuous cell lines established using prostatic cancer tissue obtained from metastatic sites from patients failing hormonal therapy. Thus, within this series of samples, the full range of the progressional state is presented. Regardless of the progressional state, the mean half-life for terminal transferase-labeled nuclei before undergoing fragmentation into apoptotic bodies ($TT_{1/2}$) was 0.5 days. Thus, in the present study, 0.5 days was used as the standardized $TT_{1/2}$ value for all subsequent calculations of K_d. The accuracy of these calculated K_d values is related to the validity of the estimated 0.5 days for the $TT_{1/2}$ value.

Using the described formulas, the K_p and K_d values were estimated for normal prostatic glandular cells, which are the cells of origin for the majority of prostatic adenocarcinomas (24). These calculations demonstrate that there is an extremely low, but balanced, daily percentage of proliferation and death of glandular cells within the prostate (*i.e.*, ≈0.2%/day). These results are consistent with previous reports that the rates of proliferation of normal prostatic glandular cells are very low (24–27). Under such maintenance conditions, the turnover time for these normal glandular cells is $500 ± 79$ days. This estimate is consistent with a previous estimate that prostatic glandular cells have an average life span of longer than 2 years using a more indirect method of calculation (28).

High-grade PIN is believed to be the major premalignant lesion for prostatic cancer (29). Early high-grade PINs are characterized by a hyperplastic increase in their K_p value with a smaller increase in their K_d values. Using these cell kinetic parameters (*i.e.*, $K_p – K_d$), it is estimated that these early high-grade PINs are growing with a mean doubling time of $154 ± 22$ days. Since one cell must undergo 26 population doublings to reach a reasonable size for detection (*i.e.*, 60 mm^3 or 5 mm × 5 mm × 5 mm; Ref. 12), ≈11 years are required for the clonal outgrowth of a high-grade prostatic intraepithelial neo-plastic cell to reach such a detectable size. It is likely, however, that this is a substantial underestimate of the actual time required for two reasons. First, the average volume of PIN in a series of 54 consecutive patients undergoing radical prostatectomy was determined to be :2000 mm^3 (30). Second, as high-grade PINs grow (*i.e.*, becoming late stage high-grade PINs), their K_d values increase to a point equaling their K_p values. This increase in the K_d value could be

explainable due to the observation that the growth of these cells is centripetal into the luminal (*i.e.*, intraepithelial) space (29). Such centripetal growth is away from the capillary blood supply in the stroma. Recent studies have demonstrated that tumor angiogenesis is a late event in prostatic carcinogenesis occurring after a hyperplastic response (31). Thus, an increasing degree of cellular hypoxia as the high-grade prostatic intraepithelial neoplastic cells continues to expand centripetally could be the mechanism for the increase in K_d in these late stage high-grade prostatic intraepithelial neoplastic lesions.

The transition of these premalignant lesions into localized prostatic cancer cells within the prostate involves no further hyperplastic increase in the K_p value. Instead, the major change during this transition is a decrease in the K_d value. This may be due to the change from centripetal to centrifugal growth of these cancer cells, which is associated with Joss of the basal epithelial layer and breakdown of the basement membrane (32). Such changes allow the centrifugally expanding cancer cells to invade the stroma, becoming closer to the capillary blood supply and thus less hypoxic. Because of the decrease in K_d, localized prostatic cancer cells within the prostate grow with an estimated mean doubling time of 479 ± 56 days. This remarkably slow rate of growth of prostatic cancer cells within the prostate has been previously demonstrated by a series of earlier studies (24-27, 33-36). At this slow growth rate, ≈39.4 years would be required for the clonal outgrowth of such a localized prostatic cancer cell to reach 1 cm^3 [*i.e.*, it takes 30 population doublings to reach 1 cm^3 (12), thus 30×479 days= 39.4 years]. Since the mean age of prostatic cancer diagnosis is 72 years (13), this suggests that prostatic carcinogenesis starts in the third to fourth decade of life. This estimate is consistent with histological data demonstrating that the frequency of high-grade PIN is highly age related, increasing rapidly between the third and fourth decade of life (37).

Due to the multistep nature and the remarkably long latent time period required to produce clinically detectable disease, prostatic carcinogenesis may be one of the most sensitive and appropriate disease processes for chemoprevention attempts. Chemoprevention is particularly appealing since the majority of available cytotoxic chemotherapies require a critical daily percentage of cell proliferation to be effective (7). Thus, the observation that the K_p values are very low (*i.e.*, <3%/day) for metastatic, as well as localized, prostatic cancer cells is entirely consistent with the clinical observations that treatment of the metastatic patients with standard antiproliferation chemotherapeutic agents have been of only limited success. These data emphasize the need for the use of chemotherapeutic agents which can activate programmed death of androgen-independent metastatic prostatic cancer cells efficiently in a proliferation-independent manner. Indeed, such agents have already been identified (8, 38).

References

1. Kyprianou N, English HF, Isaacs JT. Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. Cancer Res. 1990; 50:3748–3753. [PubMed: 2340521]
2. Isaacs JT, Lundmo PI, Berges R, Martikainen P, Kyprianou N, English HF. Androgen regulation of programmed death of normal and malignant prostatic cells. J. Androl. 1992; 13:457–464. [PubMed: 1293127]

3. Crawford ED, Eisenberger MA, McLeod DC, Spaulding J, Benson R, Dorr FA, Blumenstein BA, Davis MA, Goodman PJ. A control randomized trial of Leuprolide with and without flutamide in prostatic cancer. N. Engl. J. Med. 1989; 321:419–424. [PubMed: 2503724]

4. Isaacs, JT.; Schulze, H.; Coffey, DS. Development of androgen resistance in prostatic cancer.. In: Murphy, G.; Khoury, S.; Kiess, R.; Chatelair, C.; Denis, L., editors. Prostate Cancer, Part A: Research, Endocrine Treatment, and Histopathology. Alan R. Liss, Inc.; New York: 1987. p. 21-31.

5. Isaacs JT. Hormonally responsive vs unresponsive progression of prostatic cancer to antiandrogen therapy as studied with the Dunning R-3327-AT and G rat prostatic adenocarcinoma. Cancer Res. 1982; 42:5010–5014. [PubMed: 6215981]

6. Raghaven D. Non-hormone chemotherapy for prostate cancer: principles of treatment and application to the testing of new drugs. Semin. Oncol. 1988; 15:371–389. [PubMed: 3043671]

7. Shackney SE, McCormack GW, Cuchural GJ. Growth rate patterns of solid tumors and their relation to responsiveness to therapy. Ann. Intern. Med. 1978; 89(1):107–121. [PubMed: 666155]

8. Isaacs JT, Lundmo P. Chemotherapeutic induction of programmed cell death in non proliferating prostate cancer cells. Proc. Am. Assoc. Cancer Res. 1992; 33:588–589.

9. Tubiana, M.; Malaise, EP. Growth rate and cell kinetics in human tumors: some prognostic and therapeutic implications.. In: Symington, T.; Carter, RL., editors. Scientific Foundations of Oncology. Year Book Medical Publishers; Chicago: 1976. p. 126-138.

10. Carter HB, Marrell CH, Pearson JD, Brant LJ, Plato CC, Metter EJ, Chan DW, Fozard JL, Walsh PC. Estimate of prostatic growth using serial prostate-specific antigen measurements in men with and without prostate disease. Cancer Res. 1992; 52:3323–3328. [PubMed: 1375867]

11. Schmid H-P, McNeal JE, Stamey TA. Observation of the doubling time of prostate cancer: the use of serial prostate-specific antigen in patients with untreated disease as a measure of increasing cancer volume. Cancer (Phila.). 1993; 71:2031–2040. [PubMed: 7680277]

12. Coffey DS, Isaacs JT. Theory-prostate tumor biology and cell kinetics. Urology. 1981; 17:40–53. [PubMed: 7010755]

13. Carter HB, Isaacs JT. Experimental and theoretical basis for hormonal treatment of prostatic cancer. Semin. Urol. 1988; 4:262–268. [PubMed: 3068765]

14. Drago JR, Mostofi FK, Lee F. Introductory remarks and workshop summary. Urology. 1989; 34(Suppl):2–3. [PubMed: 2481359]

15. Steele, GG. Growth Kinetics of Tumors. Clarendon Press; Oxford: 1977. p. 56-85.

16. Cattoretti G, Becker MHG, Key G, Duchrow M, Schluter C, Galle J, Gerdes J. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB1) and (MIB3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J. Pathol. 1992; 168:357–363. [PubMed: 1484317]

17. Berges RR, Furuya Y, Remington L, English HF, Jacks T, Isaacs JT. Cell proliferation, DNA repair, and p53 function are not required for programmed death of prostatic glandular cells induced by androgen ablation. Proc. Natl. Acad. Sci. USA. 1993; 90:8910–8914. [PubMed: 8415631]

18. Martikainen P, Kyprianou N, Tucker RW, Isaacs JT. Programmed death of nonproliferating androgen-independent prostatic cancer cells. Cancer Res. 1991; 51:4693–4700. [PubMed: 1873814]

19. Kyprianou N, Isaacs JT. Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology. 1988; 122:552–562. [PubMed: 2828003]

20. Kyprianou N, English HF, Isaacs JT. Activation of a Ca^{2+}-Mg^{2+}-dependent endonuclease as an early event in castration-induced prostatic cell death. Prostate. 1988; 13:103–118. [PubMed: 2845374]

21. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Bioi., I. 1992; 19:493–501.

22. Schwarting R, Gerdes J, Niehus J, Jaeschke L, Stein H. Determination of the growth fraction in cell suspensions by flow cytometry using the monoclonal antibody Ki-67. J. Immunol. Methods. 1986; 90:65–70. [PubMed: 3711671]

23. Baisch H, Gerdes J. Simultaneous staining of exponentially growing versus plateau phase cells with the proliferation-associated antibody Ki-67 and propidium iodide: analysis by flow cytometry. Cell Tissue Kinet. 1987; 20:387–391. [PubMed: 2448031]

24. Meyers JS, Sufrin G, Maring SA. Proliferation activity of benign human prostate, prostatic adenocarcinoma and seminal vesicle evaluated by thymidine labeling. J. Urol. 1982; 128:1353–1356. [PubMed: 6185700]

25. Gallee MPW, Visser-DeJong E, Ten Kate FJW, Schroeder FH, van der Kwast. Monoclonal antibody Ki-67 defined growth fraction in benign prostatic hyperplasia and prostatic cancer. J. Urol. 1989; 142:1342–1346. [PubMed: 2478729]

26. Nemoto R, Hattori K, Uchida K, Shimazui T, Nishijima Y, Koiso K, Harada M. S-phase fraction of human prostate adeno-carcinoma studies with in vivo bromodeoxyuridine labeling. Cancer (Phila.). 1990; 66:509–514. [PubMed: 2194646]

27. Claus S, Wrenger M, Senge T, Schulze H. Immunohisto-chemical determination of age related proliferation rates in normal and benign hyperplastic human prostates. Urol. Res. 1993; 21:305–308. [PubMed: 7506462]

28. Tunn S, Nass R, Ekkemkamp A, Schulze H, Krieg M. Evaluation of average life span of epithelial and stromal cells of human prostate by superoxide dismutase activity. Prostate. 1989; 15:263–271. [PubMed: 2479940]

29. Bostwick DG. Prostatic intraepithelial neoplasia (PIN): current concepts. J. Cell. Biochem. 1992; (Suppl. 16H):10–19. [PubMed: 1644848]

30. de Ia Torre M, Haggman M, Brandstedt S, Busch C. Prostatic intraepithelial neoplasia and invasive carcinoma in total pros-tatectomy specimens: distributed, volumes and DNA ploidy. Br. J. Urol. 1993; 72:207–213. [PubMed: 8402024]

31. Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am. J. Pathol. 1993; 43:401–409. [PubMed: 7688183]

32. Gleason, DF. Histologic grading of prostatic carcinoma.. In: Bostwick, DG., editor. Pathology of the Prostate. Churchill-Livingston; New York: 1990. p. 83-93.

33. Sadi MV, Barrack ER. Determination of growth factor in advanced prostate cancer by Ki-67 immunostaining and its relationship to the time to tumor progression after hormonal therapy. Cancer (Phila.). 1991; 67:3065–3071. [PubMed: 1710538]

34. Harper ME, Gaddard L, Wilson DW, Matanhelia SS, Coon IG, Peeling WB, Griffiths K. Pathological and clinical assocations of Ki-67 defined growth fractions in human prostatic carcinoma. Prostate. 1992; 21:75–84. [PubMed: 1379364]

35. Thompson SJ, Mellon K, Charlton RG, Marsh C, Robinson MM, Neal DE. P53 and Ki-67 immunoreactivity in human prostate cancer and benign hyperplasia. Br. J. Urol. 1992; 69:609–613. [PubMed: 1379102]

36. McLoughlin J, Foster CS, Price P, Williams G, Abel PD. Evaluation of Ki-67 monoclonal antibody as prognostic indicator for prostatic carcinoma. Br. J. Urol. 1993; 72:92–97. [PubMed: 7511972]

37. Sakr WA, Haas GP, Cassin BF, Pontes JE, Crissman JD. The frequency of carcinoma and intraepithelial neoplasia of the prostate in young male patients. J. Urol. 1993; 150:379–385. [PubMed: 8326560]

38. Furuya Y, Lundmo P, Short AD, Gill DL, Isaacs JT. The role of calcium, pH, and cell proliferation in the programmed (apoptotic) death of androgen-independent prostatic cancer cells induced by thapsigargin. Cancer Res. 1994; 54:6167–6175. [PubMed: 7954463]
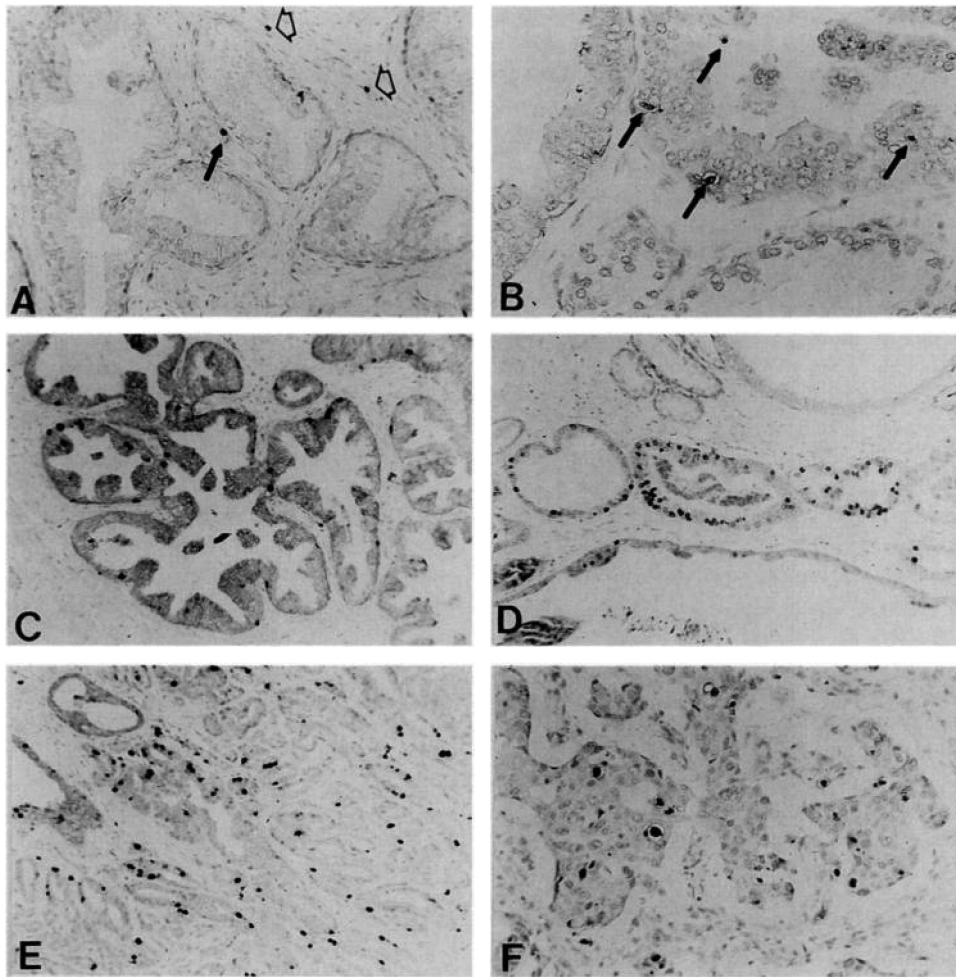
Fig. 1.
Immunocytochemical detection of prostatic cells in the proliferative cell cycle(*A*, *C*, and *E*) and undergoing cell death (*B*, *D*, and *F*). M1B1 antibody staining of proliferating cells in: *A,* normal prostatic glandular tissue; *C*, high-grade early PIN, primary prostatic cancer. Terminal transferase *in situ* end labeling of cells dying in: *B,* high-grade early PIN; *D,* primary prostatic cancer; *F,* bone metastases from hormonally untreated patients. All areas are representative of random fields except *D,* which represents a selected area of unusually high cell death, and *E,* which represents a selected area of unusually high cell proliferation. *Open arrows,* tissue in the stroma; *closed arrows,* tissue in the glandular epithelium.

Table 1

Kinetic parameters of normal and neoplastic prostatic epithelial cells

Cell type	Histological grade (Gleason sum)	% Cells		Doubling time (days)
		Proliferating/day	Dying/day (*d)	
Normal prostatic glandular epithelial cells		0.19 ± 0.03^a	0.20 ± 0.03^a	
(N = 27)		(0.05-0.30)*	(0.05-0.35)	
High-grade prostatic intraepithelial neoplastic cells				
Early (N = 10)		1.25 ± 030^c	$0.80 \pm 024^{a,c}$	154 ± 22
		(0.25-2.60)	(0.20-0.80)	
Late (N = 10)		1.80 ± 0.28^c	$1.80 \pm 0.16^{a,c}$	
		(0.20-2.50)	(0.20-4.96)	
Localized prostatic cancer cells within the prostate				
Low Gleason sum (>6) (N = 15)	5.3 ± 0.3	1.54 ± 0.17^c	1.42 ± 0.20^c	577 ± 68
	(4-6)	(0.40-2.60)	(0.25-5.25)	
High Gleason sum (>6) (N = 12)	7.9 ± 0.4	1.46 ± 0.20^c	1.32 ± 0.18^c	495 ± 56
	(7-10)	(0.80-2.85)	(0.10-3.10)	
Metastatic prostatic cancer cells from hormonally untreated patients				
Within Lymph node (N = 30)	8.1 ± 0.5	$2.90 \pm 0.30^{a,c}$	0.85 ± 0.21^c	33 ± 4
	(6-10)	{0.45-3.10)	(0.15-1.30)	
Within the Bone (N = 13)	8.2 ± 0.20	$2.04 \pm 0.29^{a,c}$	$0.76 \pm 0.16^{a,c}$	54 ± 5
	(6-10)	(0.1-2.85)	(0.10-3.60)	
From hormonally failing patients				
Within distinct nonbone sites (N = 5)	10	$2.77 \pm 0.31^{a,c}$	$2.22 \pm 0.40^{a,c}$	126 ± 21
	(10)	(0.65-3.90)	(1.50-3.65)	
Within the bone (N = 10)	9.2 ± 0.3	$2.42 \pm 0.40^{a,c}$	1.68 ± 0.38^c	94 ± 15
	(7-10)	(0.10-4.60)	(2.10-7.75)	

[a] $P < 0.05$ compared to primary prostatic cancer cells.

[b] Values in parentheses, range for particular cell type.

[c] $P < 0.05$ compared to normal prostatic glandular epithelial cells.