Quiescence in R3327-G Rat Prostate Tumors after Androgen Ablation

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ABSTRACT

Androgen ablation is frequently used in conjunction with radiotherapy in the treatment of high-risk prostate cancer. Androgen ablation-induced cell kinetic changes could result in sub-additive (increased quiescence) or supra-additive (reduction in repopulation) interactions with radiotherapy. The cell kinetic changes were studied in R3327-G Dunning rat prostate tumors grown in vivo using double thymidine analogue labeling and flow cytometry, the terminal deoxynucleotidyl transferase-mediated nick end labeling assay for apoptosis, and measurements of tumor cell numbers. Tumors grown in intact and castrate male rats were continuously labeled for various periods of time with chlorodeoxyuridine and pulse-labeled with iododeoxyuridine 8 h before tumor removal. Androgen ablation resulted in a maximal reduction in labeling index (10 to 1.6%) and an increase in potential doubling time (Tpot; 6–42 days) within 3 days, which was related to a reduction in growth fraction (65% to <10%). In contrast, the length of S-phase was minimally altered (19 to 23 h). The response to androgen ablation involved little apoptosis and no necrosis, and Tpot was approximately the same as the tumor volume doubling time. Hence, the increase in Tpot was mainly the result of a shift to quiescence, and this shift occurred with minimal cell loss. Because quiescence is usually associated with radioresistance, these cell kinetic changes suggest that a sub-additive interaction may occur for some prostate cancers when androgen ablation and irradiation are given together.

INTRODUCTION

Androgen ablation is now frequently used in conjunction with radiotherapy for the treatment of patients with high-risk prostate cancer. Preliminary evidence indicates that the combination of these modalities is superior in the short term to either treatment given individually (1–3). However, follow-up in these studies was short, and it is unclear whether androgen ablation plus radiotherapy will result in a significant survival advantage over that of radiotherapy given alone initially with androgen ablation used for salvage. Because both of these treatments result in alterations in cell kinetics and induce apoptosis, it may be possible to enhance tumor cell killing by the timing of androgen ablation relative to radiotherapy.

Apart from the induction of apoptosis in some tumors, the direct cell kinetic effects of androgen ablation on prostatic malignancies are poorly understood. In particular, there is little information regarding whether androgen ablation mainly induces a shift to quiescence, a slowing of cell cycle traverse, or an increase in cell loss (4). Should one of these mechanisms predominate, the interaction with radiation could be influenced in a sub-additive or supra-additive manner. In the absence of significant cell death due to androgen ablation, an increase in the proportion of quiescent cells should result in enhanced radioreresistance, whereas a reduction in repopulation would result in enhanced tumor radiosensitivity. In this report, the cell kinetic response of androgen-sensitive R3327-G tumors (4–6) to androgen ablation was assessed by measuring changes in LI, Tpot, and GF using thymidine analogue labeling and flow cytometry as well as AI, MI, and tumor cell density using immunohistochemical staining and histological criteria.

MATERIALS AND METHODS

Tumor Model. The R3327-G Dunning rat prostate tumor was used in the 23rd-24th in vivo transplant generations. The enzymatic digestion of the tumor, storage of the tumor cells in liquid nitrogen, and implantation of the tumor cells s.c. have been described in detail previously (5). Briefly, the cells were thawed from liquid nitrogen storage in freeze media, 10% fetal bovine serum (Life Technologies, Inc.), 10% DMSO (Sigma), and CMRL 1066 medium (Life Technologies, Inc.), and 2 x 10^5 cells were implanted s.c. in the flanks of purebred Copenhagen rats (250–300 g). Once these source tumors reached 2–3 cm, they were digested using trypsin and collagenase, and 2 x 10^5 cells were passaged into the flanks of Copenhagen rats for use in the experiments (5). The experimental tumor-bearing rats were used when the tumors were approximately 1 cm.

Castration was performed when the tumors were approximately 1 ml. Androgen restoration was investigated by implanting silastic capsules containing testosterone. The capsules were made from 0.198-cm inner diameter and 0.317-cm outer diameter silastic tubing (602–305; Dow-Corning, Midland, MI; Ref. 7). The capsules were filled with 2 cm of testosterone (Sigma) and sealed at both ends with silicone sealant (number 732; Dow-Corning). These capsules have been shown to maintain serum testosterone levels in a rat with suppressed gonadotropin levels (8).

Quantification of Apoptosis, Mitosis, and Tumor Cell Density. Portions of the tumors were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 4 μm onto slides, deparaffinized, and stained with H&E. Morphological criteria including condensed hyperchromatic nuclei and nuclear fragmentation were used to identify apoptosis (9). Twenty high-powered fields were counted with 100 cells/field. The AI and MI were calculated by taking the ratio of the number of apoptotic or mitotic tumor nuclei and dividing by the total number of tumor nuclei counted and then multiplying by 100.

One slide from each tumor was also examined by TUNEL using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD). The recommended staining procedures were followed, in principle. Mayer’s hematoxylin was used to lightly counterstain the nuclei. Cells were scored as TUNEL-positive only when they showed dark staining of an intensity equal to or greater than that of apoptotic cells in irradiated mouse intestinal crypts, which was used as a positive control. Nearly all of the TUNEL-positive nuclei seemed intact, rather than fragmented.

Tumor cell density was determined by counting the number of tumor cells/unit area in H&E-stained slides using a microscope eyepiece grid, which outlines an area of 10^-2 mm^2. A total of 10 areas were counted at x400 magnification. Nuclear dimensions (major and minor diameters) were measured on 25 tumor cell nuclei/slide using a computerized image analysis system with BioScan Options software, and the average diameter was computed. The average ratio of major:minor diameter was 1.3, which was low enough to use in the Abercrombie correction equation (10). Using the Abercrombie formula, the number of tumor cell nuclei/unit volume was calculated from the number of nuclei/unit area. Estimates of the absolute number of tumor cell nuclei/...
tumor volume are affected by shrinkage during fixation and dehydration. We assumed that all tumors had the same degree of shrinkage, so that the calculated curve would at least be representative of the relative number of tumor cells/unit of tumor volume at different times after androgen ablation.

Thymidine Analogue Labeling. The tumors were continuously labeled with CldUrd given as a continuous label for various periods of time and/or pulse-labeled with IdUrd 8 h before tumor removal. Alzet minipumps (catalogue number 2 ml1; Alza Corp., Palo Alto, CA) containing approximately 2 ml of 0.1 mM CldUrd in PBS (calcium and magnesium-free; Ref. 11) were implanted in the flank opposite the tumor for continuous labeling. In castrated rats, continuous labeling was started 3 days after castration. Pulse labeling with IdUrd involved the i.p. injection of 6 mg/kg body weight of a 30 mg/ml solution in 0.05 M Tris buffer (pH 9.5; Ref. 12). Both thymidine analogues were purchased from Sigma.

Flow Cytometric Analysis of Cell Kinetic Parameters. At tumor removal, the tissue was minced and fixed in 60% ethanol in PBS at 4°C and kept refrigerated for at least 24 h. As described previously (12), the nuclei were isolated, and DNA was denatured by incubating in pepsin for 1 h followed by 2N HCl treatment. The nuclei were then incubated with Br3 antibody (Caltag, South San Francisco, CA) to stain CldUrd, followed by phycoerythrin-conjugated antimonue IgG heavy and light chain antibody (GAM-PE; Caltag). IdUrd in the nuclei was stained with FITC-conjugated B44 antibody (B44-FITC; Becton Dickinson, San Jose, CA). The DNA was stained with propidium iodide. An EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) with 488 nm argon-ion laser excitation was used to quantify the fluorescence from phycoerythrin, FITC, and propidium iodide, as described previously (12). The machine was equipped with narrow-beam excitation optics (5 μm) for doublet analysis and exclusion.

Calculation of Cell Kinetic Parameters. The Tc, Li, and Tpot were calculated from the flow cytometric DNA versus IdUrd distributions as described elsewhere (13). IdUrd was given as a pulse-label 8 h before tumor removal. The calculations were done only for the aneuploid tumor population after fitting and subtracting out the diploid population, using MODFIT and WINLIST software (Verity Software House, Topsham, ME). Li was calculated after correction for cell division using the fν function. The RM of the distributions of labeled cells that had not divided was calculated with respect to the G1 channel (RM = 0) and the G2 channel (RM = 1). Tc was calculated from RM, and, finally, Tpot was calculated as ln2/rm

The GF was calculated from the CldUrd continuous-labeling curves using the Tc obtained from the pulse-labeling data, assuming Tc/M is 0.3 × Tc (14), and fitting the fraction of labeled aneuploid cells using a Marquardt nonlinear least squares algorithm (15). The continuous-labeling curves can be divided into three different slopes. The initial slope (t < Tc/M) corresponds to labeling of cells without division of the labeled cells. In the absence of cell loss, the slope of the continuous-labeling curve in this region should equal the Kp; however, it is difficult to resolve this region. The second region between Tc/M and Tc/M + Tc (or equivalently Tc − Tc) corresponds to labeling of proliferating cells plus division of the labeled cells and provides the most useful information. In the absence of cell loss, the slope in this region is 2Kp, which allows an accurate estimate of Kp. A third region occurs at Tc/M − Tha when all proliferating cells are labeled. The shallow slope in this region corresponds primarily to an increase in labeled P cells by division.

Kp is related to GF and Tc by Kp = ln(1 + GF)/Tc, which is approximately GF/Tc as used by Berges et al. (16) when GF is low. Tpot can also be calculated from continuous-labeling curves using Tpot = ln(2)/Kp, allowing an independent confirmation of the value obtained from the pulse-labeling curves.

The cell kinetic models were extended to account for cell loss from apoptosis. In standard cell kinetic models, p, the probability that a newly divided cell will remain proliferative, plus q, the probability that a newly divided cell will become quiescent, equals one. Apoptosis is included by assuming that there is a probability v that a newly formed nonproliferative daughter cell will become apoptotic at some later time; u = 1 − v is then the probability that the quiescent daughter cell will survive and not be lost from the population. We will assume that the cells in the compartment committed to apoptosis will be indistinguishable from other cells for a time T0 and then will be recognizable as apoptotic as defined by TUNEL staining for a time, Tc = 12 h, before fragmenting or disappearing (16).

In this general case, the cell production rate becomes

\[ \frac{\text{Kp}}{\ln(1 + \text{GF} \times \text{R})/\text{Tc}} \]

where \[ R = (e^{\text{u} \times \text{t} - 1})/(e^{\text{u} \times \text{t} - 1} + \text{AI}) \]

Under conditions in which \[ Tc > Tc > Tc, \] the continuous-labeling curves can be fitted as described above with the following equations to calculate GF and Kp.

\[ f(t) = (1 + \text{GF} \times \text{R}) \times e^{-\text{Kp} \times t} \times [1 - e^{-\text{Kp} \times (Tc \times t)}] \]

for \[ Tc < t < Tc + Tc \]

\[ f(t) = [1 - (1 - \text{GF} \times \text{R}) \times e^{-\text{Kp} \times (Tc \times t)}] \]

for \[ Tc < t < Tc + Tc \]

Furthermore, the rate of cell loss by apoptosis is

\[ Kp = \frac{\text{AI} \times \text{Kp}(e^{\text{u} \times \text{t} - 1})}{\text{E}} \]

Tds were calculated from log-transformed tumor volume data using linear regression. Tumor volumes were acquired in three dimensions with calipers using the formula \( V = \frac{4}{3} \pi \times R^3 \).

Statistics. The Student t test and nonparametric Mann-Whitney tests were used where appropriate. ANOVA was used when multiple comparisons were made.

RESULTS

The R3327-G Dunning rat prostatic carcinoma cell line is androgen sensitive, although this sensitivity varies somewhat, depending on the transplant generation used (17). The tumor growth response of tumors in the 23rd in vivo passage to androgen ablation was quantified by tumor volume measurements (Fig. 1). There was no statistically significant difference in the growth response of tumors in the 23rd or 24th in vivo passage. Fig. 1 shows the growth response to androgen ablation for tumors in the 23rd in vivo passage. Tumors in intact rats grew exponentially \( R^2 = 0.98 \) with an average Td of 6.5 ± 0.5 days (± SE) until a volume of about 7 cc was reached, at which point the rate of tumor growth slowed. Tumors in rats castrated on day 37 after tumor implant continued to increase in volume postcastration at the same rate as controls for 4–6 days, and then the growth rate declined. At day 76 (approximate tumor volume, 6–7 cc), there was a subsequent increase in the growth rate, suggesting the development of androgen insensitivity. The growth rate between days 41 and 76 was exponential \( R^2 = 0.80 \), with a doubling time of 43.0 ± 5.8 days, which was significantly slower than the growth rate for the controls \( P < 0.002, \) t test and Mann-Whitney test. After day 76, the Td for tumors grown in castrates averaged of 12.3 ± 1.5 days, which was statistically different from the initial Td after castration but was not different from the Td for tumors grown in intact rats.

The consistency of the tumors changed after androgen ablation, becoming softer and more gelatinous. In histological cross sections, most of the space was not occupied by tumor cells but by nonstaining intercellular material and stromal cells (Fig. 2). Quantification of the density of tumor cells/unit area revealed a decline that began 36–48 h after castration and continued at least until day 28 (Fig. 3, middle panel). Only a small part of the decrease in the number of tumor cells/unit area cross section of the tumor could be accounted for by a decrease in the area of the cell nuclei. Average tumor cell nuclear diameters decreased from about 7.5 μm in intact rats to 6.5 μm at 3 days after castration and 6 μm at 14 days; there were no further decreases in nuclear volume. The nuclear volume, number of nuclei/
unit area, and section thickness (4 μm) were used to calculate the number of tumor cells/unit volume. The number of tumor cells/tumor (Fig. 3, right panel) was calculated from the tumor volume linear regression equations (Fig. 1) and the number of tumor cells/unit volume. Although after castration the tumors continued to show volume increases at approximately the same rate as controls for 4–6 days and more gradual increases over the ensuing month, the number of tumor cells/tumor remained essentially unchanged. Therefore, the tumor volume increases after castration were due solely to increases in noncellular material and stromal cells (Figs. 2 and 3), and net change in cell number (cell production − cell loss) was essentially zero.

On histological review, no necrotic tumor cell areas were noted, and only a few scattered necrotic cells were observed. Moreover, the pretreatment and postcastration levels of apoptosis were low in this tumor. Using morphological criteria alone, the AI seemed to be less than 0.1%, and there was no significant difference between tumors grown in intact or castrate rats. However, using the TUNEL assay, about 0.4% of the tumor cells from tumors grown in castrate rats

![Fig. 1. Tumor volume growth curves for tumors grown in intact rats versus castrate rats. A, castration was performed when the tumors were 1 cc (demarcated by arrow). There were seven animals/group. Bars, SE. B, linear regression of log-transformed tumor volume growth curves.](image)

![Fig. 2. Histology of formalin-fixed paraffin-embedded H&E-stained sections from R3327-G tumors grown in vivo for 3 days in a castrate rat (A), 28 days in a castrate rat (B), and 28 days in an intact rat (C). A 40X objective was used with an overall magnification of ×468 after printing.](image)

![Fig. 3. Effect of androgen ablation on MI (left panel), tumor cell density (middle panel), and cell number/tumor (right panel).](image)
Table I Summary of cell population dynamics

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Intact</th>
<th>Castrate</th>
<th>Castrate and testosterone</th>
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<tr>
<td>Pulse-label parameters</td>
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<tr>
<td>LI (%)</td>
<td>9.8 ± 0.4</td>
<td>16 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Length of S-phase (h)</td>
<td>19.3 ± 0.6</td>
<td>22.6 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.9 ± 0.9</td>
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<tr>
<td>Potential doubling time (days)</td>
<td>6.2 ± 0.3</td>
<td>42.0 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7 ± 0.1</td>
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<tr>
<td>Continuous label parameters</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Length of G&lt;sub&gt;0&lt;/sub&gt; (h)</td>
<td>80.9</td>
<td>80/162&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>GF (%)</td>
<td>65.3</td>
<td>83/92&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Potential doubling time (days)</td>
<td>6.3</td>
<td>57/117&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
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<td>Other parameters</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Td (days)</td>
<td>6.5 ± 0.5</td>
<td>43.0 ± 5.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.3 ± 0.1</td>
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<td>Cell loss factor</td>
<td>0.04</td>
<td>0.02</td>
<td>&lt;0.0</td>
</tr>
<tr>
<td>AI (%)</td>
<td>0.14 ± 0.02</td>
<td>0.37 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>MI (%)</td>
<td>1.13 ± 0.14</td>
<td>0.23 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Cell density (number/field)</td>
<td>59.1 ± 2.6</td>
<td>37.0 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
</tr>
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</table>

<sup>a</sup> Mean ± SE at 3 days after castration or testosterone replacement except for the Tds, which were taken from the growth curves (in the case of castrates, the slope between 4 and 39 days was used). There was an average of six tumor-bearing animals/group.

<sup>b</sup> P < 0.05 compared to intact group, one-way ANOVA using the least significant difference and Scheffé tests where appropriate.

<sup>c</sup> Because T<sub>G1</sub> could not be determined in the castrate, the calculations assuming T<sub>G1</sub> = 80 and 162 h are shown.

The analysis of the continuous-labeling data from tumors grown in castrates was problematic, because it was not possible to make an unequivocal assignment of the break in curve (Fig. 6). Thus, the curve was fitted twice with different assumptions for T<sub>G2M</sub> + T<sub>G1</sub>. We chose T<sub>G2M</sub> + T<sub>G1</sub> times of 87 h, which was the T<sub>G2M</sub> + T<sub>G1</sub> for the tumors grown in intact rats, and 7 days (168 h) when the continuous-labeling curves seemed to flatten out. The calculated values of Tpot for these times were 57 and 117 h, respectively. These data confirm that castration induces a marked increase in Tpot. Because the Tpot obtained when T<sub>G2M</sub> + T<sub>G1</sub> was assumed to be the same as in tumors grown in intact rats showed evidence of apoptosis, whereas only 0.1–0.2% tumor cells from tumors grown in intact rats showed evidence of apoptosis (Table 1). This slight but consistent increase in apoptosis was observed even at 28 days after castration.

Tumor cell kinetics were examined by continuously infusing CldUrd for various periods of time (to estimate GF and Tpot) and pulse labeling with IdUrd 8 h before tumor removal (to estimate LI, T<sub>s</sub>, and Tpot). Examples of the bivariate DNA/CldUrd and DNA/IdUrd histograms are shown in Fig. 4. Analysis of the pulse-label histograms for tumors from control animals yielded a LI of 9.8%, a T<sub>s</sub> of 19.3 h, and a Tpot of 6.2 days (Fig. 5; Table 1). The value of Tpot was confirmed by an analysis of the continuous-labeling data (Fig. 6) that yielded a Tpot of 6.3 days.

Castration induced a dramatic decline in LI, reaching a minimal value of 1.6% by 3 days (Fig. 5). LI was subsequently maintained at this low level, indicating that a new cell kinetic equilibrium had been reached. There was a slight but significant increase in T<sub>s</sub> to 22.6 h at 3 days after castration. Tpot was estimated to be 42 days for the castrates using the pulse-labeling data. The marked increase in Tpot, which is proportional to T<sub>s</sub>/LI, was mainly the result of the decrease in LI and not changes in T<sub>s</sub>. In agreement with the flow cytometric measurements, the MI also decreased about 4-fold after castration, reaching a minimum level of about 0.3% at 3 days and remaining at that level (Fig. 3, left panel).

Fig. 4. Representative bivariate flow cytometry histograms from a tumor grown in an intact rat (A and C) and a castrate rat (B and D) 3 days after castration. A and B, results of pulse labeling with IdUrd 8 h before tumor removal. C and D, results of continuous labeling for 3 days with CldUrd beginning at the same time as castration in these same animals.
QUIESCENCE AFTER ANDROGEN ABLATION

Fig. 5. LI (A), RM (B), T5 (C), and Tpot (D) for tumors grown in intact and castrate rats determined by flow cytometric analysis. These tumors were labeled with IdUrd 8 h before tumor removal, and there was an average of five animals/group. Bars, SE.

intact rats (87 h) was closest to that obtained from pulse labeling, it seems that this $T_{G2M} + T_{G1}$ value is the better approximation. Irrespective of which value for $T_{G2M} + T_{G1}$ is assumed, the GF of the tumor is reduced in castrate rats to <10% from 65% in intact rats.

Comparison of Tpot from the pulse-labeling data with the Td allows for an estimate of cell loss factor $\Phi = 1 - Tpot/Td$. The results (Table 1) show that there seems to be negligible cell loss in the tumors in both intact and castrate rats. However, this value for the castrate rats must be interpreted with caution, because the tumor volume increase does not correspond to an increase in cell number.

The analysis in Table 2 more clearly presents the relationship between cell production rates, cell loss by apoptosis, and changes in cell number. Cell production rates in tumors from intact rats, calculated from both the pulse- and continuous-labeling data, are about 11%/day. The cell loss rate by apoptosis was much smaller, about 0.3%/day. The resulting net cell production rate of 10.8% is similar to the 10.4% derived from the cell number data. Although these numbers are in good agreement, there are possible sources of error in the calculations that should be described: (a) Al might have been underestimated, because attention was not paid to the duration of formalin fixation. Two subsequent experiments using 1-day fixation times gave values of 0.43 and 1.4% for the Al 3 days after castration; (b) if $T_A$ is less than 12 h, then the contribution of apoptosis to cell loss would be underestimated; and (c) other cell loss mechanisms in addition to apoptosis may be involved.

Table 2 also reveals that there is a major decrease in the cell production rate after castration. This is seen both using the pulse- and continuous-labeling data. The value obtained from the pulse-labeling data is probably more reliable because of the uncertainty in fitting the continuous-labeling curves after castration. For this reason, the curve fitting of the continuous-labeling castrate data was analyzed twice, assuming that $T_{G1}$ is 80 h and that $T_{G1}$ is 162 h. The cell production rates for tumors from castrate rats were 1.7%/day using pulse-labeling data and 1.2%/day using continuous labeling, assuming that $T_{G1}$ was 80 h; this continuous-labeling estimate was closest to the Tpot estimate. Because the Tpot values from continuous labeling were also in better agreement with the estimates from pulse labeling when the value of $T_{G1}$ was taken as 80 h, the results suggest that $T_{G1}$ may not have been altered significantly by androgen ablation. The net cell production rate was 0.49–0.19%/day. It can be seen (Fig. 3) that the cell number/tumor seems to decrease slightly, and this was reflected as a cell number change rate of $-0.9%/day$, although the slope was not significantly different from zero. The net cell production rate for tumors grown in castrates was about 0.5%/day versus a cell number change rate of $-0.9%/day$. This approximately 1.4% difference could be related to the error in these measurements or the other mechanisms described above. It is clear from Table 2 that the change in tumor growth rate after castration is dominated by the 10%/day decrease in cell production and is less affected by the 0.5%/day increase in cell loss.

To determine whether the reduction in LI and consequent enhancement in Tpot by androgen ablation were reversible, castrated tumor-bearing rats were given testosterone 3 days after androgen ablation, which is the time required for the new cell kinetic equilibrium. The results in Fig. 7 and Table 1 demonstrate that the LI and Tpot values had returned to the same level seen in tumors grown in intact rats by 3 days after androgen restoration. $T_S$ did not change significantly (Table 1).

DISCUSSION

The influence of androgens on prostate cancer growth is mediated through two main counterbalancing processes, cell proliferation and cell death. In an androgen-rich environment, proliferation is maxi-
The response to androgen withdrawal for R3327-G tumors differed from studies of various tumor model systems. For example, the Dunning R3327-PAP (19, 20) and R3327-G (present findings) models also had reduced cell proliferation with minimal cell loss from androgen ablation. However, the PC-82 and PC-EW tumor models, which are of human origin and are grown in nude mice, both respond to androgen ablation by decreasing the rate of proliferation and increasing the rate of cell loss (21, 22). Although there are many similarities between these cell lines, cell loss for PC-82 tumors was primarily by apoptosis, whereas necrosis predominated for PC-EW tumors.

We found using thymidine analogue labeling that the decrease in growth rate in R3327-G tumors to androgen ablation involved a decrease in the proportions of proliferating cells, which is analogous to the reduction in Ki-67 LI seen in other androgen-sensitive prostate tumors (18) and models (21, 22) described previously. Similar to most of the human tumors thus far examined (18) and the Dunning R3327-PAP tumors (19, 20), but in contrast to the PC-82 (22) and PC-EW (21) tumors, there was little evidence of cell loss in R3327-G tumors. The response to androgen withdrawal for R3327-G tumors differed from R3327-PAP tumors in that there was a decrease in the volume density of epithelial cells in R3327-G tumors (Fig. 3) that was not seen in R3327-PAP tumors (19). This can be accounted for by differences in tumor volume changes rather than cell loss. After castration, the volumes of R3327-PAP tumors remained constant, whereas R3327-G tumors maintained normal volume growth rates for 4–6 days and thereafter continued to increase in volume, albeit at much slower rates (Fig. 1). Our calculations show that despite a reduction in R3327-G tumor epithelial cell nuclear densities, the number of epithelial cells/tumor remained relatively constant, as was the case with R3327-PAP tumors (19). In R3327-G tumors, the slight increase in cell loss due to apoptosis was just enough to compensate for cell gain from proliferation.

The changes in cell population dynamics observed here for R3327-G tumors after androgen ablation required 3 days to reach a new equilibrium state; LI, Tpot, and MI continued to change during the first 3 days after castration and then leveled off. Likewise, the cell kinetics were restored to their initial state within 3 days after androgen restitution. Brandstrom et al. (20) also found that the reductions induced in LI were complete by 3 days after castration using the R3327-PAP subline; however, MI seemed to decline further between 3 and 7 days. Reductions in the labeling indices in PC-82 and PC-EW tumors seemed to be complete between 2 and 7 days after androgen depletion, but because of intertumor variability, the time could not be precisely defined (21, 22). Restoration of androgen also resulted in the reversal of this cell kinetic change in PC-82 tumors (21) and well-differentiated R3327-H tumors (23) at 3 days, although the R3327-H tumors initially showed an overshoot of the LI to twice that of tumors grown in intact rats, which finally returned to control levels by 7 days after androgen restitution. Thus, we conclude that by 3–7 days after hormonal manipulation, a new cell kinetic equilibrium is manifest in a variety of prostate cancer model systems. It should be noted that the maximal reduction in cell proliferation measured by Ki-67 index required more than a month after androgen ablation in some patients (24), implying that the time course of this response may be slower and more variable in human cancers.

The study described here provides the most complete cell kinetic characterization of any androgen-sensitive tumor to androgen ablation and restoration, using both histological and flow cytometric techniques. The new cell kinetic equilibrium reached at 3 days after androgen ablation in R3327-G tumors involved about an 8-fold reduction in the GI. These data suggest that the increase in Tpot seen Table 2 Summary of cell production and cell loss rates for tumors grown in intact and castrate rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calculated from</th>
<th>Intact</th>
<th>Castrate</th>
</tr>
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<tbody>
<tr>
<td>Cell production rate</td>
<td>Tpot (pulse label)</td>
<td>11.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Cell production rate</td>
<td>Tpot (continuous label)</td>
<td>11.1</td>
<td>1.27±0.56&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell loss rate</td>
<td>AL</td>
<td>0.27</td>
<td>0.73±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Net cell production rate</td>
<td>Difference (production – loss)</td>
<td>10.8</td>
<td>0.49±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cell number change rate</td>
<td>Tumor volume, cell density</td>
<td>10.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>−0.9 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Assuming T<sub>Gi1</sub> = 80 h.

<sup>b</sup> Assuming T<sub>Ca1</sub> = 162 h.

<sup>c</sup> From the slope of the log<sub>e</sub>-transformed and linear regression fitted cell number data, with SE shown.
after androgen ablation was principally due to a shift from a predominantly proliferating population to a predominantly resting one and was not due to changes in the rate of cell cycle transit (T_S showed very little change), although an increase in T_G1 could not be unequivocally excluded as being contributory. In the modeling of the cell cycle kinetics of tumors grown in castrates using the continuous-labeling data, the T_pot estimate obtained was closest to that from the pulse-labeling data when T_G1 was assumed to be 80 h. It seems probable, therefore, that T_G1 changed little in response to castration.

Our results and conclusions are in contrast to those of Humphries and Isaacs (4), who also studied R3327-G tumors. They reported that the primary effect of androgen ablation was an increase in the rate of cell loss, with the rate of cell proliferation remaining constant. As an index of the rate of cell proliferation, Humphries and Isaacs (4) used the amount of incorporation of [3H]thymidine into tumor cell DNA and found no significant differences between tumors grown in intact, castrated, and testosterone-replaced rats. The amount of thymidine incorporation is an indirect method of measuring cell proliferation and could be affected by changes in metabolism and intracellular precursor pool sizes. In contrast, the measurement of LI by whether or not BrdUrd is incorporated, as well as the determination of S-phase transit by the progression of labeled cells through S-phase, is independent of the amount of precursor incorporated. Humphries and Isaacs (4) reported cell loss factors (1 — T_pot/Td) of 0.36 and 0.74 for tumors grown in intact and castrate rats, whereas our calculations (Table 1) revealed the cell loss factors to be essentially zero, regardless of whether the tumors were grown in intact or castrate rats. These disparate results might be related to the use of different R3327-G tumor passages (we used an earlier transplant generation) or possibly to the way in which T_pot was calculated. Our Tds for tumors grown in intact rats were only slightly longer than theirs (6.5 versus 4.0 days), but our increase in doubling times after castration (over 6-fold) was much greater than theirs (less than 2-fold), indicating that the generation we used was much more androgen-sensitive. In the past, we documented that with increasing transplant generations, the R3327-G tumor does become less androgen sensitive (17), and they likely used a much later transplant generation.

The technique used by Humphries and Isaacs (4) to measure T_pot involved the quantification of the rate of radioactive loss from the tumor cells after an injection of [125I]IdUrd (25), whereas we used more modern flow cytometric methods using monoclonal antibodies to IdUrd and CIdUrd (26). Some of the problems associated with the [125I]IdUrd loss technique include the reutilization of label and influx of labeled inflammatory cells, but these would decrease the measured cell loss rates. Another problem is that radiolabeled nucleosides such as [125I]IdUrd may cause radiation-induced cell death and perturb cell cycle transit by blocking in G_S (27), and because there is evidence that there is an interaction between androgen ablation and radiation in androgen-sensitive tumors (28), it is possible that some enhancement in cell loss may be produced by the 125I as an artifact of the technique. The validity of our results is further supported by the consistency of the calculations from pulse- and continuous-labeling studies. For these reasons, we believe that our conclusions that cell loss was minimal and that the main action of androgen ablation was the promotion of a shift from a predominately proliferating to a predominately resting cell population are more accurate.

The androgen ablation response characteristics of R3327-G tumors seem to represent many of the same prostate cancers; however, the heterogeneous responses of human tumors should be considered when making extrapolations. Notwithstanding, the parallels between R3327-G tumors and many human prostate cancers suggest that inferences based on the results presented apply to a significant proportion of human prostate cancers. Because prostate cancer patients with a high risk of failing radiotherapy alone are commonly treated with androgen ablation before radiotherapy, the induction of quiescence could affect radiation responses. Measurements of GF with Ki-67 staining (18, 24) confirm that an increased proportion of cells are induced into a quiescent state in primary human tumors after androgen ablation. The increased quiescence in human tumors could result in a sub-additive interaction, or other mechanism(s) might compensate for this potential sub-additive interaction and enhance radiation response in this setting. What is not known is whether the susceptibility to radiation-induced cell killing (for example, via apoptosis), is altered by pretreatment with androgen ablation. Berchem et al. (29) have shown that etoposide, when combined with androgen deprivation, results in enhanced apoptosis as compared to either treatment alone. Moreover, Zietman et al. (28) have described a reduction in the radiation dose required to control 50% of tumors when androgen ablation is given with radiation. More recently, our preliminary results indicate that there is indeed a supra-additive apoptotic response when androgen ablation and single fraction radiation are combined under certain conditions (30). These data and the favorable clinical results with the combination treatment (1—3) suggest that whereas quiescence is induced as a consequence of androgen ablation, other factors act to offset this negative feature, resulting ultimately in at least an additive, and possibly supra-additive, interaction between androgen ablation and radiotherapy. Further study is needed to determine the contributions of the high fraction of quiescent tumor cells and other changes resulting from preradiotherapy androgen ablation to the interaction between the two modalities and to determine the mechanisms involved.

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Quiescence in R3327-G Rat Prostate Tumors after Androgen Ablation

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