Prostatic Tumor Regrowth after Initially Successful Castration Therapy May Be Related to a Decreased Apoptotic Cell Death Rate

Marene Landström,† Jan-Erik Damber, and Anders Bergh

Abstract

Castration of rats transplanted with the androgen-sensitive Dunning R3327-PAP prostatic tumor results initially in a reduction of tumor growth, but after some time, some of the tumors start to grow again. The relapsed, androgen-insensitive PAP tumor shows a dedifferentiated morphology. In the present study, we examined whether this androgen-independent tumor regrowth was due to an increased cell proliferation rate or to a reduction of the number of tumor cells dying by apoptosis. Nine rats (with 18 tumors) were castrated and followed for 16 to 20 weeks. Six of the tumors increased their volume markedly (relapsed), while 12 remained relatively stable (nonrelapsed). The mitotic index and apoptotic index for epithelial cells were examined by light microscopy. Tumor growth rate correlated negatively both to the apoptotic index identified by morphological criteria (RS = −0.82; P < 0.0001) and to the apoptotic index identified by in situ end labeling (RS = −0.83; P < 0.0001). The tumor growth rate percentage did not correlate to the mitotic index, and it was negatively correlated (RS = −0.62; P < 0.01) to the number of cells immunostained for proliferating cell nuclear antigen. It is suggested that one initial event during the androgen-independent prostatic tumor regrowth in the PAP relapse model might be a reduction of the number of tumor cells being depleted by apoptosis, rather than an increase of cell proliferation rate.

Introduction

PC represents the most common malignancy detected in males, and it is the second most common cause of cancer-related death in both Sweden and the United States (1, 2). Most PC is initially androgen dependent, and one standard therapy is medical or surgical castration. However, in a majority of the patients, the tumor escapes the initial beneficial effect of androgen withdrawal, and regrowth (relapse) of a now androgen-independent tumor eventually kills the patient (3, 4). Furthermore, about 20% of the PCs are already refractory to androgen withdrawal at the time of diagnosis (5). Due to these relapse phenomena, survival time has not increased in patients with advanced PC (6).

The mechanism behind the transition from an initially androgen-sensitive to an insensitive tumor remains regrettably unknown. Overgrowth of a preexisting androgen-independent tumor cell clone or a cellular adaption to growth in the absence of testicular androgens are the most common explanations (7, 8). Castration of rats transplanted with the androgen-sensitive R3327-PAP tumor results in an inhibition of tumor growth. However, after some time (10 to 40 weeks), some tumors in castrated animals begin to grow, while others remain stable for at least 1 year (9). Recent studies have shown that tumor growth rate in general is related not only to cell proliferation rate but also to the rate of apoptotic cell death (10–12). Indeed, in some experimental tumors, decreases in apoptotic cell death is the principal cause for increased growth (13–15). Interestingly, cell proliferation rate is generally low in PCs, even in low differentiated metastatic tumors (reviewed in Ref. 16), suggesting that major changes in cell proliferation rate is not the principal cause for prostatic tumor growth. Against this background, we were interested in studying whether tumor relapse after castration therapy in the Dunning R3327-PAP model could be related to changes in cell death rate.

Materials and Methods

Animals and Tumors. The Dunning R3327 PAP prostatic tumors, originating from Dr. N. Altman (The Papanicolaou Cancer Research Institute, Miami, Fl.), were transplanted to Copenhagen × Fisher F male rats, (17). This tumor subline is well characterized and androgen sensitive (18). The rats were housed under standard conditions (17) with pellet and water ad libitum. The tumor size was measured under ether anesthesia using microcalipers, and tumor volume was calculated according to the formula length × width × height × 0.5236, as described by Janik et al. (19). The percentage of TGR was calculated for each tumor as follows:

Tumor volume at end of the observation period (16–20 weeks)

Tumor volume at time for castration

We have earlier reported a significant correlation (r = 0.93) between tumor weight and calculated tumor volume (17). Four months postimplantation when the tumor volume was approximately 1300 mm3, nine rats were castrated. Castration was performed under light ether anesthesia via the scrotal route (20). Thereafter, the rats were followed with weekly tumor volume measurements.

Morphology. Randomly chosen pieces of the tumors were fixed in Bouin’s solution, dehydrated, and embedded in methacrylate plastic (Histo-Resin; LKB, Stockholm, Sweden; Ref. 20). The percentage of mitotic epithelial cells were determined by counting 5000 cells in each tumor. Parts of the tumors were fixed in 4% formaldehyde, 3% glutaraldehyde, and 0.05% picric acid in 0.05 m cacodylate buffer; the the tumors were postfixed in 1% OsO4, dehydrated, and embedded in Epon (20). One-μm-thick sections were stained with toluidine blue. The percentage of apoptotic epithelial cells in each tumor was examined at ×1000. Epithelial cells (4000) per tumor were counted in two separate measurements, and the average apoptotic index was determined. Apoptotic cells were defined as described earlier (11, 21).

Immunoperoxidase Staining for PCNA. Several randomly chosen tumor pieces were fixed in methanol and kept at 4°C for 24 h until embedded in paraffin. Sections of the paraffin-embedded material were deparaffinized and dehydrated. They were treated with 3% H2O2 for 10 minutes to block endogenous peroxidase activity. The primary PCNA antibody (Dako, Stockholm, Sweden) was applied in 1:1000 dilution for one h. The secondary antibody-biotin-labeled goat anti-mouse (Vector, Burlingame, CA) was added at a 1:200 dilution for 10 min. Avidin-biotin peroxidase complex (Vector) was added for 10 min. The sections were counterstained with hematoxylin.

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The abbreviations used are: PC, prostate carcinoma; PCNA, proliferating cell nuclear antigen; TGR, tumor growth rate; 1SEL, in situ end labeling; PBS, phosphate-buffered saline.

4 Unpublished observations.
stained with Mayer's hematoxylin for 10 min and coverslipped with water-based mounting medium. Ten different areas in each of the tumors (approximately 1000 epithelial cells) were examined, and the percentage of PCNA-positive epithelial cells were measured.

ISEL of Fragmented DNA. The protocol for ISEL staining was followed as reported by Wijsman et al. (22), as below. Several randomly chosen tumor sections were fixed in buffered 4% formaldehyde for at least 24 h and later embedded in paraffin according to routine procedures. Sections of 2-μm thickness were mounted on Starfrost precoated slides (Knittel Gläser, Braunschweig, Germany). After deparaffinization and rehydration, tissue sections were digested to enable the enzymatic incorporation of nucleotides. An optional pretreatment consisted of heating the sections in 2X SSC (0.3 m NaCl and 30 mM sodium citrate, pH 7) at 80°C for 20 min, followed by thorough washings in distilled water. Subsequently, sections were digested with 0.5% pepsin (0.9 milliAnson units/mg; Serva, Heidelberg, Germany) in HCl (pH 2) under gentle agitation at 37°C. Digestion was stopped by washing in running tapwater and then in buffer A (50 mM Tris-HCl, 5 mM MgCl2, 10 mM β-mercaptoethanol, and 0.005% bovine serum albumin) for 30 min at room temperature before developing with diaminobenzidine (Sigma). For negative controls, DNA polymerase was excluded from the nucleotide-polymerase mix. For positive controls, 3-day castrated normal rat prostate was used, since approximately 5% of the epithelial cells are known to be apoptotic in this organ (21).

After blocking endogenous peroxidase by immersion for 5 min in PBS containing 0.1% H2O2, sections were washed twice in PBS for 5 min and incubated with horseradish peroxidase-conjugated avidin (Vector) diluted in 1:100 in PBS containing 1% bovine serum albumin and 0.5% Tween 20 for 30 min at room temperature before developing with diaminobenzidine (Sigma).

For negative controls, DNA polymerase was excluded from the nucleotide-polymerase mix. For positive controls, 3-day castrated normal rat prostate was used, since approximately 5% of the epithelial cells are known to be apoptotic in this organ (21).

Statistics. Values are expressed as the median and the 25th and 75th percentiles. Correlation was expressed by the Spearman rank test and the linear correlation coefficients (23). P < 0.05 was considered as statistically significant.

Results

Tumor Growth and Morphology. Castration resulted in an initial retardation of the tumor growth rate in all of the 18 Dunning R3327-PAP prostatic tumors studied (as reported previously; Ref. 9). After various times, some of the tumors started to grow again, while others remained growth inhibited. Five of the tumors relapsed after 10 weeks, and one tumor relapsed at 18 weeks with a median tumor volume at the time for castration. P < 0.05 when compared to nonrelapsed tumors. The percentage of PCNA-positive tumor epithelial cells correlated negatively to TGR (RS = -0.62; P < 0.01), but the percentage of mitotic cells did not correlate to the TGR percentage of PCNA-positive tumor epithelial cells are known to be apoptotic in this organ (21).

In sections from nonrelapsed tumors, several apoptotic epithelial cells were identified (Fig. 3A). In relapsed tumors, only few apoptotic tumor epithelial cells were observed (Fig. 3B; Table 1). TGR was negatively correlated to the number of ISEL-positive cells (RS = -0.83; P < 0.0001). The number of morphologically apoptotic cells in the relapsed tumor was decreased when compared to nonrelapsed tumors, and the apoptotic index was negatively correlated to TGR (RS = -0.82; P < 0.0001). There was a strong positive correlation between the number of morphologically apoptotic and ISEL-positive tumor epithelial cells (RS = 0.90; P < 0.0001).

Discussion

Castration initially inhibits the growth of the Dunning R3327-PAP prostatic tumor. However, after several weeks, some of these tumors start to grow again in the absence of testicular androgens (9). The growing tumors are histologically more dedifferentiated than those that remain more stationary (9). This alteration of an initially androgen-sensitive adenocarcinoma to an androgen-insensitive and partly dedifferentiated tumor strongly resembles the clinical situation in which prostatic carcinoma patients, treated with androgen withdrawal, experience a progression of their disease and the tumor often is histologically dedifferentiated (9, 24, 25). In this study, we demonstrate that the increased tumor growth during the early transition to androgen-independent growth is appar-

**Table 1** The values of TGR (%), mitotic index (MI), PCNA index, apoptotic index (AI), ISEL-positive cells, and the ratio between MI and ISEL of Dunning R3327-PAP prostatic adenocarcinoma growing in castrated rats

<table>
<thead>
<tr>
<th>TGR (%)</th>
<th>MI (%)</th>
<th>PCNA index (%)</th>
<th>AI (%)</th>
<th>ISEL index (%)</th>
<th>MI/ISEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapsed tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N = 6</td>
<td>324.5b</td>
<td>0.3</td>
<td>2.35b</td>
<td>0.45b</td>
<td>0.18b</td>
</tr>
<tr>
<td></td>
<td>(296-411)</td>
<td>(0.22-0.38)</td>
<td>(1.86-2.82)</td>
<td>(0.34-0.56)</td>
<td>(0.11-0.28)</td>
</tr>
<tr>
<td>Nonrelapsed tumors</td>
<td>126.5</td>
<td>0.5</td>
<td>5.1</td>
<td>1.08</td>
<td>1.1</td>
</tr>
<tr>
<td>N = 12</td>
<td>(104-142)</td>
<td>(0.37-0.64)</td>
<td>(3.0-6.9)</td>
<td>(0.91-1.24)</td>
<td>(0.81-1.48)</td>
</tr>
</tbody>
</table>

* Median values are given with 25th and 75th percentiles within parentheses. n = number of tumors studied. We define relapse as a regrowth of the tumor more than 200% of the tumor volume at the time for castration.

* P < 0.05 when compared to nonrelapsed tumors.
REDUCTION OF APOPTOSIS IS RELATED TO TUMOR RELAPSE

600
500
400
300
200
100
0.5 1.0 1.5 2.0
Fig. 2. The apoptotic index for tumor epithelial cells are plotted against the individual TGR.

y = 338.8 - 149.8 x
Rs = -0.82 (p<0.0001)
r = -0.59 (p<0.01)

Fig. 3. Sections from a nonrelapsed (A) and a relapsed (B) Dunning R3327-PAP prostatic tumor. × 1000. Note the differences in morphology between the two sections. The number of epithelial cells has markedly increased in the relapsed tumor (B) when compared to the nonrelapsed (A) tumor. Note also the almost complete absence of apoptotic cells in the relapsed tumor when compared the number of apoptotic cells (arrows) in the nonrelapsed tumor. Apoptotic cells are fulfilling the diagnostic criteria for apoptotic cells as earlier described with fragmented and condensed nuclei as reported earlier (11, 21).

tently not primarily caused by an increased cell proliferation. The mitotic index (slightly reduced) was not correlated to TGR, and the PCNA-labeling index negatively correlated to TGR. PCNA is normally expressed in the nucleus during the DNA synthesis phase of the cell cycle from late G1 to early mitosis (26, 27), but recent studies show that it may also be expressed in noncycling cells (28). The mechanisms behind the decreased PCNA labeling in relapsing tumors is thus difficult to interpret. The present suggestion that the early transition to androgen-independent growth in the Dunning R3327-PAP tumor is not caused by increased cell proliferation is in line with the observation that there is apparently no major increase in cell proliferation in recurrent human PCs (29). On the other hand, the cell proliferation rate is correlated to histological grade and also to some extent to prognosis in PC patients (reviewed in Ref. 16). This finding and our previous observation in this Dunning relapse model of a weak positive correlation between TGR and mitotic index in tumors that have grown more than in the present study (9) suggest that the cell proliferation rate is of importance for prostatic tumor growth. The present study does, however, suggest that other factors may be more
important during the early transition to androgen-independent tumor growth.

In contrast, the early transition from androgen-dependent to androgen-independent tumor growth in the Dunning R3327-PAP tumor could be related to a decrease in the numbers of cells dying by apoptosis. The observation of a decreased apoptotic index was confirmed by two different methods, by morphological criteria with the light microscope and by ISEL. Recent studies have shown that tumor growth rate in general is determined by the balance between cell proliferation and cell death (10, 11). In the liver, it has been shown that tumor promoters may act by inhibiting apoptosis (14, 15). Moreover, it has been hypothesized that tumor relapse in general could be caused by tumor cells that have become defective in the program of apoptotic cell death (12). The present findings in the prostate support this hypothesis. The cell biological mechanisms behind the transition from androgen-dependent to androgen-independent cell growth are unknown. One current opinion is that it is caused by autocrine secretion of growth factors from the tumor cells (8); other studies suggest that it is caused by repopulation of the tumor by an androgen-independent clone of tumor cells that was present already prior to castration (7). The present study offers a third possibility. It could be related to defects in the apoptosis program, possibly by induction of apoptosis-inhibiting genes. Indeed, there are now data suggesting that the expression of bcl-2, an oncprotein which prevents programmed cell death (33–34), is increased in androgen-independent human PCs (33, 34). It also appears that the expression of bcl-2 could be increased by androgen ablation treatment in the normal rat prostate and in the prostate and its association with emergence of androgen-independent prostate malignancies. Arch. Pathol. Lab. Med., 111: 845–847, 1987.


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