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Analysis of Changes in Rat Prostate Carcinoma Following Hormone Deprivation

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The R3327-H model of prostatic adenocarcinoma was employed for the study of the cellular changes that occur during induction, regression, and recurrence of prostate cancer after endocrine therapy. The present study was designed to compare the glandular and stromal elements of the relapse phase with the histologically distinct early and intermediate phases of tumor progression. Morphometric analysis revealed significant differences between all three groups in the percentages of total tumor occupied by the epithelial component. At all three time periods, high-power inspection of autoradiograms prepared after incubation of the tissues with radioactive dihydrotestosterone revealed large cells in the stroma, especially in the intermediate phase. Immunohistochemistry further revealed evidence of invasion across the prostatic acinar basement membranes by similar cells. These studies lead the authors to postulate a mechanism by which hormone-independent cells in the epithelium repopulate the stroma, causing a recapitulation of the original morphology of the tumor in the postremission period. They propose that prostate tumor response to estrogen therapy can be operationally defined in three phases: involution, rebound, and relapse. They infer that further knowledge of the timing of these phases may permit early selective use of specific therapeutic strategies which will be able to balance the clinical risk with the known behavior of the neoplasm during progression of the disease. (Am J Pathol 1987, 128:566–572)

HUMAN prostate tumors are generally composed of two neoplastic populations of cells: androgen-dependent and androgen-independent. Thus, androgen deprivation therapy, which kills only the former group of cells, is palliative, rather than curative.[1] Prostate tumors that recur after such therapy are generally insensitive to further hormone therapy and are subsequently treated with chemotherapeutic agents. Perhaps because of this, the death rate from prostate cancer has not decreased significantly over the past four decades.[1]

Surprisingly, little attention has been given to the histopathologic and biochemical changes that occur in prostate tumors during the period of initial remission. Most of the early work on the histology of prostate carcinoma following endocrine therapy[2–5] was accomplished in the period shortly after the introduction of estrogen therapy by Huggins et al in 1941.[6] These studies identified many of the common individual cytologic changes that occurred after administration of the synthetic estrogen diethylstilbesterol (DES). However, it has not been possible to define changes in heterogeneous tumor populations during the time course of remission and relapse, because of the small number of samples which were obtained from each individual. Information remains difficult to obtain, because no current justification exists for

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repeated sampling of patient tissues during the period of time when the primary tumor is "in remission."

We reasoned that "relapse" of prostate carcinoma must actually begin some time before this becomes clinically manifest. Thus, we employed an animal model of prostate carcinoma to identify early morphologic events that might signal the end of the period of remission.[7-9]

The R3327-H tumor model (H), which can be grown in isogenic Copenhagen rats and in athymic mice, permits the evaluation of tumor responses to a variety of steroids and anti-tumor agents. It is a slow-growing, androgen-dependent tumor with a doubling time of over 20 days.[7] In addition, it forms a well-differentiated tumor that contains receptors for estrogens and progestogens.[10,11] When the H tumor is grown in orchiectomized rats or mice, it ceases to proliferate for a period of eighty days and then resumes its growth. A new, androgen-insensitive nonmetastatic cell line (HI) results from this manipulation.[7,12]

We employed an *in vitro* autoradiographic method that identifies specific cells in which steroids accumulate in the nucleus, thus permitting analysis of clonal heterogeneity within tissues at the cellular level.[13] This morphologic technique can define changes in the percentage and distribution of receptor-containing cells within the tumor mass. Using this technique, we were able to study the hormone-sensitive large neoplastic epithelial (LNE) cell population present in the stroma of this tumor, which we describe in a companion paper.[14]

In the present study, the histologic, morphometric, and endocrine changes that occur in the Dunning R3327H tumor during intermediate and late phases of tumor progression were characterized. It was noted that many cells in the epithelium and stroma of tumors from castrated males retained high levels of androgen receptor, even though tumor growth had become independent of androgen stimulation. The significance of this finding is discussed. A multiphasic model of tumor relapse is proposed.

Materials and Methods

Tumor Transplantation and Organ Culture

Pieces of Dunning R3327-H rat prostate adenocarcinomas 2–4 mm in diameter were implanted subcutaneously in the flank of 30 adult male NIH I (T-cell-deficient) athymic mice.[15] Approximately 3 months later, when tumors had attained a volume of approximately 1 cu cm, the animals were separated into three treatment groups: 1) a control group (n = 8), bearing hormone sensitive H tumors (H); 2) a long-term-orchiectomized group (n = 10), which was allowed to survive for 90 days after orchiectomy, a time at which the tumor becomes hormone-insensitive (HI)[7] and growth returns to the control rate ("relapse"); and 3) a group allowed to survive 30 days after orchiectomy (n = 6), an intermediate time during which the tumor is in transition from hormone-sensitive to hormone-insensitive status (THI). All mice were anesthetized with 0.05 ml of a 1 : 1 mixture of acepromazine and ketamine hydrochloride and orchiectomized via a scrotal incision route.

In order to clear endogenous androgen from the circulatory system prior to autoradiography, the control (H) tumors were harvested 1 day after orchiectomy, prior to the initiation of postorchiectomy morphologic changes. H tumors from control animals that had survived 180 days were compared with these smaller tumors for determining whether the morphology of the tumor from intact mice was affected by age or tumor size.

Tumors from all groups were minced into pieces 1–2 mm in diameter in plastic culture dishes containing Dulbecco's phosphate buffer (0.1 M, pH 7.25). Minced samples of viable tumor tissue were incubated for 4 hours in RPMI 1640 culture medium with tritiated dihydrotestosterone (^{3}H-DHT) (210 Ci/mmol; New England Nuclear, Cambridge, Mass) in a shaker water bath at 37 C to permit binding of labeled androgen receptors. Competition studies were performed by the addition of 100 nM unlabeled estradiol or testosterone propionate to the incubation medium. After incubation, the tissues were washed for 4 hours with hourly changes of 10 ml RPMI 1640 medium containing 3.5% (wt/vol) bovine serum albumin (Fraction V, Sigma, St., Louis, Mo) to provide high capacity binding sites for removal of unbound steroid.

Autoradiography

The tumor pieces were placed on a tissue base of minced rat liver mounted on brass cryostat stubs. Tumors were snap-frozen in liquid isopentane at −70 C and stored in liquid nitrogen before processing for autoradiography according to the methodology of Stumpf et al[16] as modified by us.[14] Frozen sections (4 μm) were cut in a cryostat at −35 C and thaw-mounted onto slides that had been precoated with Kodak NTB-3 autoradiographic emulsion. One slide each was developed on Days 4, 8, 12, 21, 30, and 60 of incubation. Tissues were stained with methyl green–pyronin and examined for the presence of cells with silver grains over cell nuclei. For morphometry, photographs of the slides were prepared with a 16× Zeiss apochromat lens on a Leitz Orthoplan microscope.

Microscopy of autoradiograms was performed with a Zeiss Photomicroscope I utilizing 25× and 40× Zeiss planapochromat oil immersion lenses. In slides with sections that showed marked morphologic heterogeneity, care was taken to photograph representative samples of the whole section. Thus, if one area showed decreased cellularity as compared with an adjacent area, a portion of the hypocellular area was included in the photographic field in approximate proportion to its contribution to the entire section.

Morphometric Analysis

Photographs were coded prior to evaluation. A dot matrix grid was superimposed on the photographs for quantification of tumor morphology.[17] The matrix contained 294 fine points on an 8 × 8-mm grid printed on a 5 × 8-inch acetate sheet. The size of the grid was adjusted to ensure that the dots were spaced far enough apart so that only one dot would fall on a given element of tissue morphology. Dot size was small enough to prevent a single dot from falling in two tissue compartments simultaneously. The dot matrix was superimposed over each photograph, and the area directly under each point was then scored as "epithelium," "stroma," or "lumen." On less than 10% of the slides, the tissue sections were not as large as the photographic field, and therefore fewer than 294 points were scored. The relative proportion of each component was expressed as a percent of the total points counted. Statistical analysis was performed.

Statistical Analysis

For pairwise comparisons within epithelium, stroma, and lumen, Wilcoxon rank sum tests were used; exact P values were obtained from published tables.[18] For the comparison of more than two groups within epithelium, lumen, and stroma, the Kruskal–Wallis test was used. This test is the multisample extension of the Wilcoxon rank sum test. The P value given for this test is based on a chi-square approximation. The criterion for significance was set at $P<0.05$. Computations were undertaken with PROC NPAR1WAY software licensed by the Statistical Analysis System (SAS Institute Inc., Cary, NC).

Immunohistochemistry

Autoradiograms prepared for combined autoradiography and immunohistochemistry were immunostained after the method of Keefer et al.[19] Prior to photographic development, slides were placed in a 4% paraformaldehyde solution (4 C, pH 7.0) for 60 seconds. Subsequent to photographic development, slides were washed for 5 minutes in running tap water and then placed in 0.05 M Tris buffer (pH 7.6) for 5 minutes followed by treatment with 1% normal sheep serum (NSS) (5 minutes) to block nonspecific binding sites prior to application of the antibody bridge.

A double peroxidase–antiperoxidase method[20] was used for immunocytochemical demonstration of laminin. Rabbit antilaminin antiserum (Bethesda Research Laboratories, Bethesda, Md) was used at a concentration of 1 : 100 in Tris buffer supplemented with 1% NSS. The primary antiserum was applied overnight at room temperature. For each set of immunostained slides, one control slide was processed for which normal rabbit serum was substituted for the primary antiserum to control for nonspecific deposition of reaction product.

Results

Tumor Growth in Nude Mice

Tumors implanted subcutaneously in nude mice reached a reproducibly measurable size of 1 cu cm^3 after 3 months (doubling time approximately 21 days). Castration at that time stopped proliferation of the tumor for a period of 80 days. Thus, the tumors from castrate males were not significantly larger than they had been 3 months previously. However, tumors from noncastrated control animals continued to grow, attaining volumes three to five times the starting volume. As it was unclear whether the additional volume might cause morphologic differences, a second set of noncastrated tumor-bearing control animals (180 days from transplantation) were compared with the original control tumors harvested at the start of the experiment (90 days from transplantation). With the exception of occasional focal necrosis, no morphologic differences were observed between 90-day and 180-day control tumors.

Morphometry

Morphometric analysis was performed on prostatic tumors. Comparison of neoplasms from the 30 day (THI) and 90 day (HI) groups with the noncastrated control (H) animals revealed significant differences in the percentages of total tumor area occupied by the epithelium (Table 1). The glandular epithelium from 1-day castrated males occupied the highest percentage of tumor area (24.59 ± 1.08). The epithelial area decreased significantly in the THI group (13.55 ± 1.67, $P<0.01$ versus control) and then returned to near control levels in HI tumors (20.47 ± 1.50, $P<0.01$ versus THI), although this percentage was still significantly lower than that of the control group ($P<0.05$).

Table 1—Percentages of Tumor Volume Occupied by Different Tissue Components

	Epithelium*	Stroma*	Lumen*
Control (intact male; n=8; hormone-sensitive, H tumor)	24.59 + 1.08†	65.37 + 1.36	10.04 + 0.62
30-day orchiectomized (n=6; transitional hormone-insensitive, THI tumor)	13.55 ± 1.67	76.87 ± 4.00	9.58 ± 2.79
90-day orchiectomized (n=10; hormone-insensitive HI tumor)	20.47 ± 1.50	62.80 ± 4.55	16.73 ± 4.21

*See text for details.
†Mean ± standard error of the mean.

At the same time, the percentage of tumor occupied by stroma varied inversely with the epithelial component. Stroma occupied the smallest percentage of the control group (65.37 ± 1.36), increased in the THI group (although this point fell short of being statistically different from the control group; 76.87 ± 4.00; $P = 0.061$) and returned to near control levels in the HI group (62.80 ± 4.55; the HI group was significantly different from the THI group; $P < 0.05$). The H and HI groups were not significantly different in the percentage of tumor occupied by stroma.

The range of sizes of luminal profiles in this model was too great to permit valid statistical comparisons between control and experimental groups. Wide variations in luminal area existed between samples and within the same groups.

Light Microscopy

The three groups were histologically distinct (Figures 1–3). The H group (Figure 1) displayed the typical features of adenocarcinoma, with glands lined by a moderately well differentiated epithelium containing stratified cuboidal cells varying in thickness from one to two cell layers. The lumens were often filled with an eosinophilic proteinaceous secretory fluid. Furthermore, the stroma was characterized by an admixture of reactive fibroblasts, macrophages, and a small number of mixed benign inflammatory cells surrounding a rich vascular supply. Larger epithelial-type cells with prominent nuclei were also appreciated within the stroma.

In contrast, the tissue samples from THI tumors revealed significant changes in tumor morphology from that seen in the first group (Figure 2). The epithelium was more frequently stratified and the thickness of the epithelium was increased. In addition, the volume of the acinar secretions appeared reduced, although not so statistically. The staining characteristics of the fluid were similar to the control group. Most notably, the area of the tumor occupied by stroma was markedly increased, with a diminished cellularity in many areas of stroma. Interestingly, the large cells which were observed throughout the stroma of control tissues appeared to be restricted to an area in close proximity to the circumference of the acini (Figures 2, 4, and 5).

The histologic appearance of the HI tumor had changed dramatically (Figure 3). Acini now appeared larger and more numerous, and their epithelial linings appeared stretched and attenuated. Some acini were very large in volume, exceeding the luminal sizes observed in any other group. The epithelium generally consisted of a single layer of "squamous" epithelial cells. The stroma in this group was more uniformly cellular than that of the THI group. The obvious ringing of acini by large stromal cells was no longer a prominent feature of the tissue (Figure 3).

Autoradiography

At all three time periods, high-power inspection of autoradiograms prepared following incubation of the tissues with radioactive DHT revealed large epithelial cells in the vicinity of acini. Excess unlabeled DHT prevented specific labeling, but excess unlabeled estradiol did not block ^{3}H-DHT uptake by tumor cells (data not shown). Labeling of these epithelial cells was especially evident in the THI group (Figures 4 and 5). Laminin immunohistochemistry was performed to identify the basement membranes of acini. In Figure 4, the dark line surrounding acini represents the diaminobenzidine reaction product formed in the location of the laminin protein. Several large cells embedded within the laminin matrix appear to be exiting from the acinus into the adjacent stroma. Other cells of the same type are present in the vicinity of the acini, apparently no longer attached to the laminin. Similar cells are observed throughout the stroma in all three groups studied (Figures 1–3). A more detailed analysis of this phenomenon is addressed in the companion manuscript.[14]

Discussion

Dunning R3327-H rat carcinoma cells, when grown in rats or nude mice, produce tumors with prominent hypercellular stroma. We have previously shown that nuclei of a prominent subpopulation of these stromal cells have a high concentration of receptors for DHT and 17β-estradiol (E_2) and that their

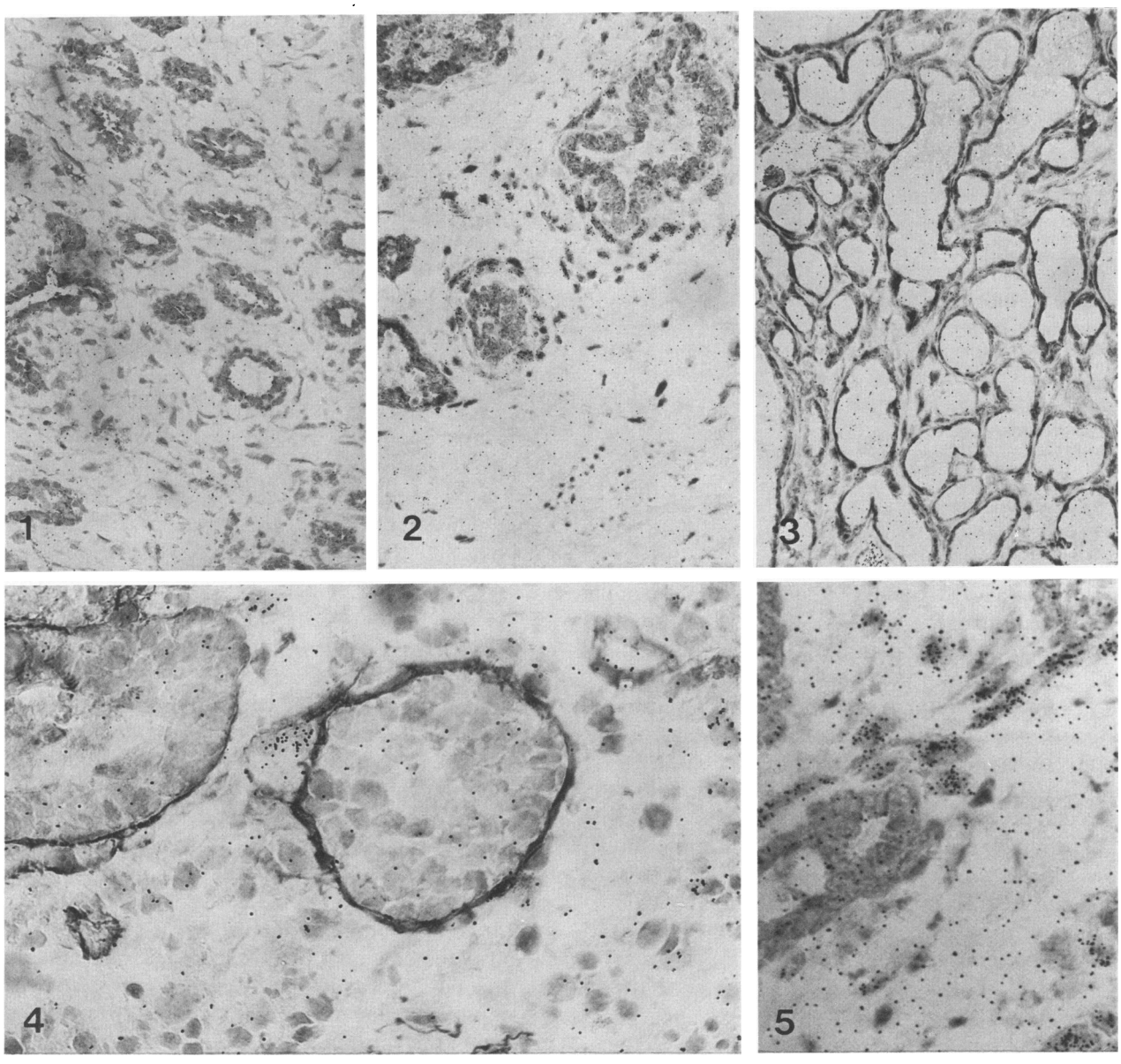

Figure 1—R3327-H prostate adenocarcinoma. Control tissues demonstrate a moderately well differentiated neoplasm. The acinar epithelium consists of stratified cuboidal cells surrounding eosinophilic proteinaceous fluid within the lumen. Connective tissue in the H stroma is admixed with large numbers of prominent large neoplastic epithelial (LNE) cells. (Methyl green–pyronin [MGP] stain, ×200) **Figure 2**—Tumors from animals orchiectomized 30 days (THI) reveal a thicker epithelium surrounding the neoplastic glands. That fraction of the tumor area composed of stromal elements is increased, with diminished stromal cellularity. LNE cells, which are heavily labeled and thus appear darker in this illustration, appear to be located primarily in the vicinity of surviving acini. (MGP, ×200) **Figure 3**—Tumors from animals orchiectomized 90 days (HI) reveal repopulation of the stroma by LNE cells. Acinar epithelium appears flattened. (MGP, ×200) **Figures 4 and 5**—Autoradiograms prepared following overnight incubation of THI tumors with radioactive DHT reveal large labeled LNE cells in the vicinity of acini. **Figure 4**—The basement membrane is identified by its laminin immunoreactivity (dark linear staining around the periphery of acini). A single LNE cell with numerous nuclear silver grains is present within this matrix. Note attenuation of laminin immunoreactivity on the stromal side, suggesting degradation of basement membrane by the cell. (MGP, ×500) **Figure 5**—After separation from the acinar epithelium, LNE cells (identifiable by increased concentrations of nuclear silver grains) appear to migrate into the relatively cell-free extracellular matrix left behind after death of the androgen-sensitive cell population. (MGP, ×300)

origin is epithelial.[14] In the present investigation these large neoplastic epithelial (LNE) cells were present at all times studied. When tissues were stained immunohistochemically for the basement membrane glycoprotein laminin, epitheloid cells were identifiable both in the stroma and on the luminal side of acinar basement membranes. This is remarkable in light of the autoradiographic evidence that the cells have a higher androgen binding capacity than the acinar epithelial cells, and thus would have been expected to be adversely affected by castration. In the early stages of transformation, invasive LNE cells may not possess increased numbers of androgen receptors. This could account for the variability in the number of silver

grains present over nuclear profiles. Alternatively, the clonal heterogeneity of the surviving cells may account for this variability.

It has been demonstrated that relapse of the H tumor following orchiectomy is a result of selection of cells which were already insensitive to androgen, rather than adaptation of existing cells to the changed endocrine environment.[21] Clearly, the androgen-dependent clones in this model die after orchiectomy. We have compared the morphology of these tumors at three time points after orchiectomy in an attempt to further characterize the processes of "remission" and "relapse" in this system.

By 30 days after orchiectomy, profound morphologic changes have occurred. A significant decrease in epithelial cellularity is the most obvious change, but autolytic and phagocytic processes are not discernible. Many LNE cells can be seen in close proximity to the acini from which they arise. When autoradiograms from this period are studied at high magnification, it is observed that these cells retain the capacity to specifically bind androgen. At this intermediate stage, then, it appears that a subpopulation of cells has survived the effects of orchiectomy, representing a clone which is still capable of binding androgen, but which is not dependent on androgen for survival.

On the basis of this study, we suggest that there are three phases of prostate tumor response to estrogen therapy, which we have termed the involutional, rebound, and relapse phases (Table 2). Cell death and the subsequent remodeling of the tumor bed occur rapidly after orchiectomy. Because this process had already been completed by the 30-day time point we selected, and because the doubling time of the tumor is approximately 21 days, we postulate that the involutional phase, during which phagocytosis of dying cells must take place, requires approximately 21 days to complete. At this point only the hormone-independent (HI) clones remain.

A prominent feature of the morphology of the relapsed HI tumor was the amount of secretory fluid in the acini. Microscopic examination of the HI tumor revealed many highly distended luminal profiles in which epithelial cells had a more "squamous" appearance than cells in the control H tumor. Although the range of acinar profiles was too great to make acinar size a significant criterion for statistical differentiation of the two tumor states, this finding suggests that seminal fluid secretion is unregulated in this system. Thus, it follows that not only cellular proliferation, but also protein synthesis and secretion, in the relapsed tumor may have become disconnected from their normal hormonal regulatory mechanisms.

We have previously reported that LNE cells also have a high estrogen binding capacity.[13] Mobbs[22] has demonstrated that in this tumor model estrogen therapy induces greater quantities of progesterone receptor (PR), a classical criterion for functionality of the estrogen receptor (ER) in female mammals. Young et al. have reported a case in which ER levels were elevated in a prostate tumor following DES therapy.[23] The frequency with which such results occur is not known, since ER levels are not frequently requested for late-stage prostatic disease. This suggests that DES therapy for prostate carcinoma, in addition to its well-known effect of suppressing androgen secretion, may sensitize the tumor cells that survive androgen deprivation to further therapy with other endocrine hormones or hormonally related chemotherapeutic agents, such as Estracyt.[24]

Table 2—Proposed Phases of Tumor Response to Androgen Deprivation Therapy in the Dunning R3327-H Prostate Tumor

Involutional phase
The bulk of androgen dependent cells die.
Estrogen induces both its own receptor and the progesterone receptor in estrogen-sensitive cells.
Autolytic enzymes are released by these hormone-responsive cells into the stroma, causing additional death of glandular cell elements.
Reticuloendothelial cells are attracted to the involuting tumor bed.
Rebound phase
Autolytic enzyme release and host white cell accumulation are reduced.
Additional extracellular matrix becomes a template for tumor proliferation.
A period of rapid cell proliferation occurs among androgen-independent cells.
Relapse phase
Proliferating tumor cells refill the available matrix left behind by the death of androgen-dependent cells.
Cell division returns to its former slower rate.
Increases in tumor mass occur primarily because of prolonged cell survival, rather than rapid proliferation.

In the early years of endocrine therapy, Fergusson and Franks reported 150 cases in which multiple samples of the tumor were obtained.[2] These authors did not have enough serial samples to determine interim hormonal effects on individual tumors. However, their observation that the postestrogen-treated tumors which eventually killed the patients were very similar in morphology to the original tumors is similar to our findings in the present study. It may be that many hormone-responsive tumors which survive endocrine therapy contain clones of cells which have retained significant quantities of hormone receptor and continue to be sensitive to the effects of androgen. This is corroborated by the evidence that androgen therapy of hormone-treated tumors often causes flare-up of metastases.[25]

These initial studies indicate that alternative approaches to the management of prostate carcinoma therapy might be developed in which different therapeutic strategies would be utilized in each of the three

phases we have suggested. The presence of hormone-sensitive cells within relapsed tumors following DES therapy suggests that the application of antiestrogens[26] or chemotherapeutic agents linked to hormonal carriers[23] would be effective.

Late-stage prostate carcinoma is currently treated by a two-step approach, with either estrogen therapy or orchiectomy being administered first and chemotherapy being reserved for patients who fail on hormone therapy. Because patients are often elderly and likely to die of natural causes prior to relapse of the disease, chemotherapy is rarely administered at the time of discovery of the disease. This is likely to continue to be the therapy of choice in such circumstances; however, for younger patients, combination hormonal and chemotherapy may be acceptable if experimental findings indicate a probable therapeutic advantage. Isaacs[27] and Rosenberg et al[28] have demonstrated that the coadministration of chemotherapy with endocrine therapy confers additional survival advantage upon animals bearing tumors of the H model. It may be that the precise timing of chemotherapy to coincide with the early stages of hormonally induced involution will be shown to be of significant advantage in treatment of human carcinoma as well.

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