Adaptation versus Selection as the Mechanism Responsible for the Relapse of Prostatic Cancer to Androgen Ablation Therapy as Studied in the Dunning R-3327-H Adenocarcinoma

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ABSTRACT

The Dunning R-3327-H rat prostatic adenocarcinoma is a well-differentiated, slow-growing, serially transplantable tumor of spontaneous origin. When intact male rats bearing such an exponentially growing H-tumor s.c. are castrated, tumor growth abruptly stops, demonstrating the initial androgen sensitivity of this tumor. Eventually, however, after an extended period, the tumor invariably relapses and once again appears to grow exponentially. At the time of relapse, the tumor is no longer androgen sensitive but has irreversibly progressed to a completely insensitive state. The mechanism responsible for this irreversible progression has been demonstrated by fluctuation analysis not to be due to environmentally induced adaptation of initially androgen-dependent H-tumor cells to a new androgen-independent state. Instead, the progression is due to the basic heterogeneity of the original H-tumor (i.e., it is composed of a mixture of preexisting clones of both androgen-dependent and androgen-independent tumor cells). Following castration, only the preexisting clones of androgen-independent tumor cells are able to continue exponential growth; the androgen-dependent tumor cells stop proliferating and die. Thus, androgen ablation creates a host environment in which the androgen-independent tumor cells have a highly selective growth advantage over the androgen-dependent cells. Eventually, with time, this selective growth advantage results in a tumor which is completely composed of androgen-independent cells. It is the continuous proliferative growth of these androgen-independent tumor cells which leads to the relapse phenomenon.

INTRODUCTION

The normal adult prostate requires a continuous supply of androgen to maintain both its cell number and secretory activity. Prostatic cancer often retains a similar androgen requirement for its growth, and therefore, this form of cancer is often highly responsive to androgen ablation therapy. While approximately 60% to 70% of all men with prostatic cancer treated by androgen ablation do respond, indicating that indeed their cancers are initially androgen sensitive, essentially all of these men eventually relapse to a state unresponsive to further antiandrogen therapy (4). What is the mechanism for this relapse phenomenon wherein an initially androgen-sensitive prostatic cancer progresses following androgen ablation to an androgen-insensitive state?

One possibility is that prostatic cancers are initially composed of tumor cells that are homogeneous at least in regard to their requirement for androgenic stimulation of their growth. Following androgen ablation, some of these androgen-dependent cells, under environmental pressure, randomly adapt to become androgen independent. These androgen-independent cells, once formed, could proliferate without the requirement for androgenic stimulation and thus eventually repopulate the tumor producing a relapse to androgen ablation. In such an explanation, the changing host environmental conditions following androgen ablation are assumed to be critically involved in inducing the adaptive transformation of an initially androgen-dependent to an androgen-independent tumor cell (environmental adaptation model). In contrast to this adaptive process where the changing hormonal environment is assumed to play a direct inductive role, an alternative explanation is possible in which the role played by the changing hormonal environment following androgen ablation is only indirect (environmental selection model). It is possible that initially prostatic cancers are heterogeneous, at least in regard to their androgen requirements for growth, being composed of preexisting clones of androgen-dependent and androgen-independent cells. Androgen ablation, in such a context, would result in the death of only the androgen-dependent cells without affecting the continuous growth of the androgen-independent ones. These independent cells would continue to proliferate following androgen ablation such that, even if these androgen-independent cells initially represented only a small fraction of the starting tumor, they would eventually not only completely replace any tumor loss due to the death of the androgen-dependent cells but progressively expand the tumor population producing the relapse phenomenon. In order to experimentally determine if either the environmental adaptation or selection model is indeed responsible for the relapse to androgen ablation therapy, the transplantable Dunning R-3327-H rat prostatic adenocarcinoma was utilized as a model. This tumor was chosen since it is a well-differentiated, slow-growing, androgen-sensitive prostatic cancer which mimics many of the properties of human prostatic cancer (2).

MATERIALS AND METHODS

Tumor Inoculation Protocol. The Dunning R-3327-H tumors were always less than 2 cu cm in total size when used for donor inoculation purposes. These small tumors were initially excised, and the entire tumor was minced with scissors. The minced tissue from a single tumor was then mixed with 20 ml of a 1% collagenase solution (type I; Worthington, Freehold, N. J.) The collagenase solution was made with HBSS containing 1.26 mm calcium and 0.85 mm magnesium obtained from Studied in the Dunning R-3327-H Adenocarcinoma1

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from Grand Island Biological Co. (Grand Island, N. Y.). This mixture was then incubated at 37° for 1 hr. After this initial incubation, the mixture was filtered with a 0.5-mm gauze wire mesh strainer (Cellector; Beltco, Vineland, N. J.); this filtrate contained very few viable tumor cells and was discarded. The residue was washed on the strainer with 100 ml of calcium- and magnesium-free HBSS, and the wash was discarded. The washed residue was then gently pressed through the strainer with the aid of a small flat glass spatula. The strainer was then washed with 20 ml of calcium- and magnesium-free HBSS. The material passing through the screen was then diluted with an additional 10 ml of calcium- and magnesium-free HBSS containing 3% Pronase (Calbiochem-Behring Corp., La Jolla, Calif.). The entire mixture was then incubated for 30 min at 37°. Following this second incubation, the entire mixture was centrifuged at 500 × g for 5 min. The resulting cell pellet was then gently resuspended in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co.), and the entire wash cycle was repeated. After the final wash, the H-tumor cells were resuspended to a dose of 3 × 10^6 viable cells/ml as determined by the standard trypan blue exclusion technique. The resultant cell suspension (0.5 ml) was then injected s.c. into the flank of the recipient animal. Besides this standard cell suspension method of tumor inoculation, some animals, where indicated, were alternatively inoculated s.c. in the flank with 10-mg trocar pieces of solid H-tumor. Both types of inoculations (tumor cells or pieces) at the doses used produced 1-cu cm tumors in intact male rats with essentially identical times (approximately 140 days). In addition, the histology and tumor-doubling times for these tumors were identical regardless of the method of inoculation.

Animals. All animals used in this study were F1 hybrid males of the cross between purebred male Copenhagen and female Fischer rats. These F1 hybrid males were inoculated with tumors at 60 to 80 days of age. Castration, when performed, was via the scrotal route using ether anesthesia. Exogenous androgen replacement, when given to the indicated castrated rats, was as daily s.c. injections of 2 mg of testosterone propionate (Steraloids, Wilton, N. H.) dissolved in 0.2 ml of propylene glycol.

Determination of Tumor-doubling Time, Apparent Percentage of Injected Cells Growing at Day 0, and Time Required for Tumors to Grow to 1 cu cm. Individual tumor dimensions were serially measured for all tumors using calibrated microcalipers at various times following tumor inoculation. These dimensions were then used to calculate the individual tumor volumes according to the formula (length × height × width × 0.5236) described by Janek et al. (3). The paired serial observations of days post-tumor inoculation (x) and the corresponding log tumor volume (y) at each of the respective time points measured were then individually entered for each tumor separately as (x,y) pairs of conjugate variables into a Texas Instrument Model TI-55 calculator preprogrammed to determine, by means of standard linear regression analysis, the equation for the line of best fit. Using this equation, the time required for individual tumor inoculums to grow to 1 cu cm was calculated as was the individual slope (Δ log tumor volume/day) and y-intercept (log tumor volume at Day 0) for each tumor. The individual tumor-volume-doubling times (in days) were then calculated by dividing the log 2 by the slope of the regression line determination for each tumor. The apparent percentage of injected tumor cells which initially grow at Day 0 was determined by dividing the apparent number of cells growing at Day 0 by the total number of starting viable tumor cells injected (1.5 × 10^6) and multiplying by 100. The apparent number of injected tumor cells which grow at Day 0 was itself determined by multiplying the apparent tumor volume (cu mm) at Day 0, as determined by the antilog of the y-intercept of the regression line for each tumor, by the number of tumor cells per cu mm. The number of tumor cells per cu mm was determined by dividing the total DNA per cu mm of tumor tissue, measured according to the method of Coffey et al. (1), by the DNA content per cell, measured by assaying known (hemocytometer) numbers of tumor nuclei for their DNA content.

RESULTS

Androgen Sensitivity of R-3327-H Tumor. When 1.5 × 10^6 viable H-cells are injected s.c. into intact adult male F1 rats, H-tumors become palpable approximately 40 to 50 days post-inoculation in essentially 100% of the animals. Once palpable, the growth of these tumors is continuous as revealed by the plot of the tumor volume versus days post-tumor inoculation (Chart 1). This linear growth curve can be replotted as the log of tumor volume versus days post-tumor inoculation to demonstrate that, between 50 and 180 days, the growth of the H-tumor in intact male rats is exponential as shown by the fact that the tumor-doubling time of 21 ± 6 days is constant during this period (Chart 1). In addition, from this semilog plot it is possible to calculate that the apparent percentage of the injected H-cells which initially grew at Day 0 in intact male hosts is 91 ± 4%. If castrated male rats are inoculated with 1.5 × 10^6 viable H-cells at Day 0 and immediately begun on continuous daily testosterone propionate injections (2 mg/day), the H-tumor again becomes palpable after 40 to 50 days and grows exponentially with a tumor doubling time of 23 ± 7 days (data not shown). In this androgen-supplemented case, the apparent percentage of the injected H-cells which initially grew is 95 ± 9%, a value identical with that seen in intact male rats. In direct contrast to the situation when H-tumor cells are injected into either intact male rats or castrated males maintained with exogenous androgen, if castrated male hosts are injected with 1.5 × 10^6 viable H-cells and no exogenous androgen is given, tumors are not palpable at 40 to 50 days. Instead, approximately 100 days are required before they appear (Chart 1). Once palpable, however, these tumors too grow exponentially with a constant 25 ± 8-day tumor-doubling
time (Chart 1). While this growth rate is essentially identical to that observed for the tumors grown in intact hosts, the apparent percentage of the injected cells which initially grew at Day 0 in castrated male rats is only 24 ± 10%, a value only one-quarter of that for tumors grown in intact hosts. Taken together, these data demonstrate that the H-tumor is not strictly speaking androgen dependent, since approximately 25% of the initially injected H-cells can grow even in androgen-ablated castrated male hosts. These data demonstrate that the H-tumor is, however, highly androgen sensitive, in that approximately 60% to 70% of the injected cells do not grow in castrated animals without androgen.

Relapse of the H-Tumor to Castration. At 150 days postinoculation of 1.5 x 10⁶ viable H-tumor cells into intact male hosts, the tumors, approximately 1 to 2 cu cm in volume, are growing exponentially and are histologically uniform, well-differentiated adenocarcinomas with essentially no area of necrosis (Fig. 1A). The tumors are composed of prominent well-developed acini, the lumen of which are filled with periodic acid-Schiff reagent-positive secretions indicative of mucopolysaccharides. In addition, each tumor acinus is surrounded by well-developed stromal elements. If intact rats bearing such exponentially growing H-tumors are castrated at Day 150, the tumor abruptly stops its exponential growth and for approximately 60 days does not increase its volume (Chart 1). This initial response to androgen ablation again indicates that indeed the H-tumor is highly androgen sensitive. This sensitivity is revealed not only by the cessation of progressive tumor volume growth but also by the histological appearance of the tumor during this androgen ablation responsive period. One month following castration, not only is there an increase in the proportion of the tumor which is necrotic but, even in areas that appear well preserved grossly, there are now large relatively acellular areas in which very few tumor acini are found (Fig. 1B). These areas are essentially composed of only the stromal element of the tumor (i.e., collagen and fibroblasts). These areas, depleted of tumor acini, are adjacent to large areas where tumor acini are perfectly maintained with no evidence of acinar involution or cellular death. The acini in these well-maintained areas are fully secretory as revealed by the presence of periodic acid-Schiff's reagent-positive secretions in their lumens. Approximately 2 months following castration, this initial response to androgen ablation is subsequently followed by a renewal of proliferative growth, indicating that the tumor has relapsed to androgen ablation (Chart 1). The semilog plot of tumor volume versus days postinoculation (Chart 1) demonstrates that the growth of this relapse tumor is exponential as illustrated by its constant tumor doubling time of 23 ± 8 days between Days 200 and 275 postinoculation. The histological picture of the tumor when it has relapsed to androgen ablation, as judged by its renewed exponential growth, is now uniformly well differentiated with very few areas depleted of tumor acini (Fig. 1C).

Complete and Irreversible Androgen Insensitivity of the Relapse Tumors. If the tumors which develop following relapse to castration (termed HI tumors) are used for further serial transplantation, the tumors which they produce are completely androgen insensitive. This can be documented by the fact that the tumors produced by inoculation of 1.5 x 10⁶ viable HI tumor cells into intact, castrated, or castrated rats supplemented with daily testosterone propionate injections all had histologies identical with that shown in Fig. 1C, and all tumors grew at identical rates regardless of the androgen status of the host (Chart 2). The doubling time for these transplanted HI tumors is 22 ± 3 days in intact hosts, 24 ± 5 days in castrated hosts, and 26 ± 5 days in castrated hosts treated with exogenous testosterone propionate. In addition, the calculated apparent percentage of the injected HI tumor cells growing at Day 0 is between 85% and 90% regardless of the androgen status of the host. These observations demonstrate that once the initially androgen-sensitive H-tumor relapses following androgen ablation, the relapse tumor is completely and irreversibly androgen insensitive.

Mechanism for Progression of Androgen-sensitive H-Tumor to the Insensitive HI Tumor following Castration. To determine whether the androgen ablation-induced progression of the androgen-sensitive H-tumor to the androgen-insensitive HI tumor is due to adaptation or selection, fluctuation analysis was performed on the H-tumor. If the H-tumor is initially heterogeneous, being composed of substantial areas of androgen-dependent and -independent tumor cells, then small trocar pieces of the H-tumor of identical size (10 mg) taken at random throughout the tumor should vary widely in their individual ratios of androgen-dependent to -independent cells. Therefore, if such trocar pieces are used to individually inoculate rats, each animal will receive a constant number of total tumor cells composed, however, of a highly variable number of androgen-independent cells. If allowed to grow in intact animals, all such trocar inoculums should grow to produce tumors of 1 cu cm volume with essentially identical times since the total number...
of starting cells in each case is identical and, under such conditions, both androgen-dependent and -independent cells grow equally well. That this last statement is true is demonstrated in Charts 1 and 3 which illustrate that, in intact rats given injections of 1.5 x 10⁶ viable H- or HI tumor cells, both take approximately 140 to 150 days to grow to 1 cu cm, with the apparent percentage of initially injected cells which grow at Day 0 being essentially identical for both H- and HI tumors. In direct contrast to the consistency in the time required for trocar pieces to grow to 1 cu cm in intact rats, individual trocar pieces, when inoculated into castrate rats, should require widely fluctuating times to grow to 1 cu cm if the original H-tumor is heterogeneous, since each trocar piece would have varying starting numbers of androgen-independent cells. If the H-tumor is not heterogeneous but is composed of uniformly androgen-dependent cells, and castration simply induces the random adaptive transformation of some of these cells, then individual trocar pieces of identical cell number should each have the same frequency of this adaptive transformation, and the time required to grow to 1 cu cm should be very similar for all trocar pieces. Therefore, the fluctuation in the time required for individual trocar pieces to grow to 1 cu cm in castrated rats can be used to differentiate between these 2 different mechanisms for relapse. As a control to judge the normal base-line fluctuations in growth response due to technical problems of tumor passage, the entire H-tumor remaining after removal of the trocar pieces was dissociated into tumor cells as outlined in “Materials and Methods.” These cells are then carefully mixed so that each cell suspension inoculation will have the same number of starting viable tumor cells as that of the trocar pieces. When these uniform cell suspensions are injected into intact versus castrated rats, the time required for the tumors to grow to 1 cu cm should be much longer in the castrated hosts. However, the fluctuation in the times to grow to 1 cu cm between individual castrated animals inoculated with these uniform cell suspensions should be small since each receives a constant number of viable cells of identical average composition. Therefore, the magnitude of the fluctuation in the time required for these uniform cell suspension inoculations to produce 1-cu cm tumors in castrated rats can be used to define the upper limit of the random fluctuation expected due simply to technical problems of tumor passage only. The data in Chart 3 demonstrate the actual fluctuation in the time required for tumors to grow to 1 cu cm in 10 intact and 10 castrated rats individually inoculated with either trocar tumor pieces or uniform tumor cell suspensions. For graphic purposes, each of the tumors was assigned an individual number on the basis of increasing time for the respective tumor to reach 1 cu cm postinoculation. In this way, the variation in time between Tumors 1 and 10 graphically illustrates the full range of fluctuation seen for each group. A horizontal line would indicate identical growth rates for all 10 samples. In contrast, an increased slope reflects the degree of fluctuation in the samples. Examination of Chart 3 reveals that the fluctuation for the 10 intact animals inoculated with trocar pieces or uniform cell suspension is identical; the mean time to 1 cu cm for the trocar piece inoculations being 130 ± 18 days as compared to 135 ± 16 days for uniform cell suspension inoculations in intact rats. In direct contrast, Chart 3 reveals that the fluctuation for the 10 castrated rats inoculated with trocar pieces as compared to castrate rats inoculated with cell suspensions is not identical. Clearly, the fluctuation in the time required to produce 1-cu cm tumors in the 10 castrated animals inoculated with trocar pieces is much larger than that seen for the 10 castrated rats inoculated with comparable tumor cell suspensions. The mean time for the castrated group inoculated with trocar pieces to produce tumors of 1 cu cm is 250 ± 80 (S.D.) days which is, however, essentially identical to the mean value of 240 ± 25 days found for the castrated group inoculated with cell suspensions. The more than 3-fold increase in the standard deviation of the mean seen in the castrated group inoculated with trocar pieces (±80) as compared to that of the castrated rats inoculated with cell suspensions (±25) again demonstrates that this large fluctuation is not simply due to technical problems of tumor passage but to the basic nature of the H-tumor. These results are not compatible with the idea that the H-tumor before castration is homogeneously androgen sensitive and that androgen ablation induces the new development postcastration of androgen-insensitive tumor cells. If this had occurred, the fluctuation in the time required to produce 1-cu cm tumors should have been very similar between the castrated animals inoculated with trocar pieces as compared to those inoculated with uniform cell suspensions.

As an additional control to judge the validity of such fluctuation analyses, similar analyses were performed using trocar pieces versus uniform cell suspensions of the androgen-insensitive HI tumor. The results of these studies (Table 1) demonstrated that there was no significant difference in either the mean time to produce 1-cu cm tumors or the standard deviation in intact or castrated hosts inoculated by either method. This
Table 1
Fluctuation analysis on the androgen-insensitive HI tumor

<table>
<thead>
<tr>
<th>Host status</th>
<th>Type of tumor inoculated*</th>
<th>Time for HI tumor to reach 1 cu cm (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Trocar pieces</td>
<td>155 ± 24</td>
</tr>
<tr>
<td>Intact</td>
<td>Uniform cell suspension</td>
<td>142 ± 22</td>
</tr>
<tr>
<td>Castrated</td>
<td>Trocar pieces</td>
<td>150 ± 25</td>
</tr>
<tr>
<td>Castrated</td>
<td>Uniform cell suspension</td>
<td>140 ± 15</td>
</tr>
</tbody>
</table>

* Six animals/group.

consistency further demonstrated both the usefulness of fluctuation analysis and the complete androgen insensitivity of the HI tumor.

DISCUSSION

When intact adult male rats bearing the androgen-sensitive Dunning R-3327-H tumor are castrated, the H-tumor appears to abruptly stop its exponential growth and for 60 days does not increase its tumor volume. After this initial response to androgen ablation, the androgen-sensitive H-tumor irreversibly progresses to a completely androgen-insensitive state. It is this irreversible progression to an androgen-insensitive state which is the basis for the relapse to androgen ablation. The mechanism responsible for this irreversible progression is not adaptation of initially androgen-dependent H-tumor cells to a new androgen-independent state induced by a changing host environment following androgen ablation. Instead, the progression to an androgen-insensitive state is dependent upon the initial heterogeneity of the H-tumor, the tumor being composed of a mixture of preexisting clones of both androgen-dependent and -independent cells. Androgen ablation creates a host environment in which the preexisting clones of androgen-independent cells have a highly selective growth advantage over the androgen-dependent cells. Following castration, only these androgen-independent cells continue to exponentially proliferate; the androgen-dependent tumor cells stop proliferating and die. For a time following castration, the continuous loss of tumor volume due to the death of androgen-dependent tumor cells is balanced by the addition of tumor volume due to the continuous exponential growth of the preexisting androgen-independent cells. During this time, the net tumor growth appears static. This situation of static tumor growth is only temporary, however, for eventually all the androgen-dependent cells are gone, and the continued exponential growth of the androgen-independent tumor cells is no longer balanced by the death of the dependent tumor cells. When this happens, the tumor volume begins to exponentially grow again indicating that relapse has occurred. At relapse, the tumor is now completely and irreversibly androgen insensitive. It is the continuous growth of these androgen-insensitive tumor cells which eventually kills the host animals. In hundreds of animals bearing the R-3327-H tumor, castration alone has never cured any animal. In this regard, the H-tumor mimics very closely the clinical response seen in Stage D human prostatic cancer, where too, initial response to androgen ablation therapy is almost universally followed by subsequent relapse.

The similarity in clinical response between the H-tumor and human prostatic cancer emphasizes the strong possibility that human prostatic cancer too may be heterogeneous with regard to its androgen sensitivity. It is therefore becoming clear that future attempts to treat human prostatic cancer must consider the strong possibility that prostatic cancers are composed of phenotypically heterogeneous subpopulations of tumor cells which exist even before treatment has begun. Initial therapies based, therefore, solely upon a single approach (e.g., hormonal manipulation) would appear to have little chance of success since they affect only a portion of the tumor population. The use of additional modalities (e.g., radiation, chemotherapy, etc.) specifically targeted at the androgen-insensitive tumor cells early in the treatment of prostatic cancer, when combined with androgen ablation, would thus appear to be highly promising.

REFERENCES

Fig. 1. Histologies of the R-3327-H tumor before, during, and after response to androgen ablation. A, tumor growing exponentially in intact male rats before castration; B, tumor not growing 1 month following castration; C, relapse tumor growing exponentially again 2 months following castration. × 40.
Adaptation \textit{versus} Selection as the Mechanism Responsible for the Relapse of Prostatic Cancer to Androgen Ablation Therapy as Studied in the Dunning R-3327-H Adenocarcinoma

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