Tumor Progression in Serial Passages of the Dunning R3327-G Rat Prostatic Adenocarcinoma: Growth Rate Response to Endocrine Manipulation

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ABSTRACT

Serial passages of the poorly differentiated, androgen-sensitive R3327-G prostatic adenocarcinoma were used to study the progressive changes that occur in tumor growth rate and androgen sensitivity. Different in vivo transplant generations (21st to 28th) were compared. The tumor doubling and animal survival times resulting from the implantation of the 21st to 22nd generation (21–22G) tumor cells in intact male rats were significantly greater than those resulting from the implantation of 23–28G tumor cells. The most dramatic difference between early (21–22G) and late (26–28G) tumor generations, however, was in androgen sensitivity. The 26–28G tumors displayed androgen sensitivity only when implanted into animals castrated 2 to 7 days previously. Tumors grown in the pretreated castrates grew at a significantly slower rate than those in intact rats and the pretreated castrates had longer survival times than the intact rats. When 26–28G tumors were allowed to grow in intact rats to approximately 1 cu cm and then the rats were castrated, no significant difference in the growth rate between these tumors and tumors grown in intact rats was observed. In contrast, the androgen sensitivity of 21–23G tumors could be demonstrated, regardless of whether treatment was started before or after implantation. The fact that androgen sensitivity was still evident under certain conditions in late-generation R3327-G tumors demonstrates that the basic mechanism involving androgen response was still present, although functioning at a much reduced level.

INTRODUCTION

The best approach to the treatment of prostatic carcinoma via endocrine manipulation remains controversial (8). There are 2 distinct philosophies. Based on evidence from the Veterans Administration Cooperative Urological Research Group (2, 23), many patients with Stage C or Stage D disease are not treated until they become symptomatic (i.e., bone pain). The rationale for this late treatment is that there is little difference in survival between patients treated early in the course of their disease (asymptomatic) and later (symptomatic). The deciding factor, therefore, is the palliation of symptomatic disease. On the other hand, some urological oncologists favor the initiation of treatment soon after the diagnosis of Stage C or Stage D prostatic cancer is established (20). The rationale here is to reduce the tumor burden and determine the efficacy of treatment as early as possible, and, where applicable, delay the transition from Stage C to Stage D. Tumor progression is a factor which concerns the decision of when to treat asymptomatic, later stage, prostatic cancer patients with endocrine manipulation that has not been addressed. The role that tumor progression has on the endocrine responsiveness of prostatic cancer is examined in this study.

The serial passage of tumors in animals often leads to the loss of cellular organization, loss of functional characteristics, and increased growth rate (9, 12, 13–16). Foulds (7) termed these events tumor progression and considered them the result of the inadvertent selection of mutant cells. The inadvertent selection that occurs during repeated in vivo passages may also occur during sustained tumor growth in men. Should the mutation-selection process of tumor progression involve a reduction in prostatic tumor androgen sensitivity (i.e., progression towards hormonal autonomy), then response to endocrine manipulation, and hence patient survival, would be adversely affected by delaying treatment.

Over the last several years, we have studied the growth kinetics of the poorly differentiated, fast-growing, androgen-sensitive R3327-G rat prostatic adenocarcinoma (17–19, 21). This spontaneous, transplantable tumor was originally found by Dunning (6) in a section of the dorsal lobe of the prostate from a Copenhagen rat. The R3327-G line has been very stable in terms of growth rate and androgen sensitivity for 20 transplant generations. Recently, we observed that after 26 to 28 transplant generations in vivo, androgen sensitivity was diminished.

The important implications concerning the effect of tumor progression on the response of prostatic carcinomas to endocrine manipulation and the widespread use of the Dunning tumor lines prompted us to characterize the effects of tumor progression on the R3327-G line. In this paper, we compare the growth kinetics and androgen sensitivity of different R3327-G in vivo transplant generations and document the changes that occur in these parameters.

MATERIALS AND METHODS

Tumor Implantation. Male Copenhagen × Fischer F1 rats (200 to 250 g) were implanted with 1 to 2 × 10⁷ viable enzyme-dispersed tumor cells as described previously (17, 18). Briefly, tumor tissue was dispersed into single cells by placing finely minced tumor in a trypsinization flask containing 20 g of tissue per 100 ml of 0.25% trypsin, 0.02% EDTA, and 0.02% collagenase in phosphate-buffered saline. The suspension was stirred at room temperature, and the dispersed cells were decanted at 20-min intervals. The trypsin activity was reduced by the addition of Connaught Medical Research Laboratories Medium 1415 containing 10% calf serum.

No significant differences in growth rates or time to palpability were observed when either 1 × 10⁷ or 2 × 10⁷ cells were implanted. The cells used for implantation ranged in age from the 21st to the 29th in vivo transplant generation.

The protocol involved the thawing of viably frozen enzyme-dispersed
R3327-G cells (17, 18) and injection s.c. into intact recipient male carriers. The tumors were allowed to grow to 3 to 5 cu cm and were then excised, enzyme dispersed, and reimplanted at 1 to 2 x 10^2 cells/rat for the experiments. Thus, if the 22nd generation (22G) was stored frozen in liquid nitrogen, the tumors in the carriers were designated "23G," and the tumors used in the experiment were designated "24G." Cells thawed from liquid nitrogen were always grown in carriers prior to use in an experiment, because the cells from liquid nitrogen took 1 to 2 weeks more to form palpable tumors than those from carriers and were available in limited quantities.

**Treatment.** Orchiectomy was performed through a transverse scrotal incision (21) under ketamine hydrochloride with 10% acepromazine maleate anesthesia. TP^2 (The Upjohn Co., Kalamazoo, MI) was diluted in sesame seed oil and administered s.c. 5 times per week in 0.25 ml per rat.

**Tumor Volume.** Tumor volume was approximated using 3-dimensional measurements by the formula 0.5236 x L x W x H (4). The tumor volume data were log transformed and fitted using the Gompertz equation (22)

\[ \ln N = \ln N_0 + \frac{B}{A} + \frac{B}{A}(e^{-\frac{T}{A}}) \]  

(A)

where \( N \) is tumor volume (cu mm), and \( T \) is time (days) post-tumor implantation. The variables \( N_0 \) (initial tumor volume), \( A \), and \( B \) were solved for using the Marquart analysis (Plot 50; Statistics, Vol. 4; Tektronix, Beaverton, OR) as described previously (18). The doubling times were calculated from the formula

\[ Td = \ln2/\left[ \frac{B}{A} - A \ln N/N_0 \right] \]  

(B)

where \( Td \) is the doubling time based on volume (22). The values of the constants \( N_0 \), \( A \), and \( B \) in Equation B were taken from the results of Equation A. The doubling times of each of the individual tumor volume curves were averaged by treatment group and compared statistically using the Student t test.

**RESULTS**

**Effect of Castration on Growth Rate**

Chart 1 shows the differences in tumor growth and response to castration between 23rd and 26th generation R3327-G tumors (23G and 26G, respectively). The tumors were allowed to grow to 0.5 to 1.0 cu cm before the animals were castrated. The data show that the growth rate of 23G tumors was significantly reduced by castration after 1 week. In contrast, castration had no effect on the growth rate of 26G tumors. This lack of response to castration under these conditions was consistently observed with 26G and later generation tumors. The results suggest that the androgen sensitivity of the R3327-G subline was diminished or lost in the later transplant generations (26G and above).

There are several possible mechanisms that could be involved in the progression to hormonal autonomy observed in Chart 1. Possible explanations for the changes in the response characteristics of R3327-G tumors to castration are categorized below and defined in terms of the mechanisms that may be operating.

**Increased Time to Response.** The results in Chart 1 could be explained as an increase in the time required for 26G tumors to respond to castration. If such an increase in time to response occurred independently of any changes in extent of response, then the growth rate of 26G tumors grown in castrates would eventually be reduced to the same level as 23G tumors grown in castrates. The mechanism would involve a slowing in the diminution of the growth rate of the androgen-sensitive cells in response to castration.

**Decreased Extent of Response.** In this case, the reduction in growth rate in response to castration is diminished, but not eliminated. The growth curves in Chart 1 indicate that a decrease in the extent of response did not occur in 26G tumors, unless this change was paired with an increase in the time to response. For example, if 26G tumors showed only a decrease in the extent of response, then the growth rate would have been between that of 23G tumors grown in intact controls and 23G tumors grown in castrates.

A decrease in the extent of response can be manifested by 2 mechanisms. (a) The androgen-sensitive cells might give rise to less sensitive cells, and/or (b) the ratio of androgen-insensitive cells to androgen-sensitive cells might increase. Both mechanisms would result in a reduction in the inhibition of tumor growth by castration. A decrease in the extent of response could be operative, in conjunction with, or independently of, changes in time to response.

**Elimination of Response.** The results in Chart 1 could be explained as a complete loss in the ability of 26G tumors to respond to castration. The major difference between a decrease in the extent of response and an elimination of response is in degree. Elimination of response is the end point for the reduction in the extent of response, and the same mechanisms apply. The most likely mechanisms would be the total loss of the androgen-sensitive population (11). It is also possible, though, that the response of the androgen-sensitive cells to castration is reduced to undetectable levels, yet these cells are still capable of response to more stringent methods of androgen deprivation (e.g.,

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*The abbreviation used is: TP, testosterone propionate.*
high-dose diethylstilbestrol treatment) (18). To examine the
effect on tumor implant.

The data in Chart 2 illustrate the long-term effects of castration
on R3327-G growth. Castration in these experiments was
not performed prior to tumor implant, so that by the time the tumors
were palpable, the cells had been exposed to an androgen-deprived
environment for 2 to 4 weeks (depending on tumor
generation). This permitted the growth response to castration to
stabilize prior to the onset of tumor volume measurements.

Under these conditions, tumors grown in castrates grew at a
significantly slower rate than did tumors grown in intact controls
for all tumor generations tested (Chart 2). These data demon-
strate that, in the later transplant generations (26G and above),
androgen sensitivity was retained; the response of the androgen-
sensitive cells to castration was not eliminated. In fact, the
growth rate of 27G tumors grown in castrates was approximately
the same as that of 22G tumors (Table 1). Therefore, the change in
androgen sensitivity observed in Chart 1 was due to an
increase in the time required for R3327-G tumors to respond to
castration.

A major difference between the 22G and 27G tumors shown
in Chart 2 was in the time to palpability when grown in intact
male rats. The average time to palpability for 21-22G tumors
grown in intact rats (3 to 5 weeks) was always longer than for
23-28G tumors (1 to 2 weeks). The doubling time data in Table
1 show that 22G tumors grown in intact rats grew at a signifi-
cantly slower rate than analogous 27-28G tumors. The differ-
ences in the time to palpability appeared to be related to the
growth rate than to the number of tumor cells surviving the initial
implantation procedure. In an attempt to confirm this, initial tumor
volumes (N0) were calculated from the Gompertz equation (22).
The results, however, were inconclusive due to the high vari-
bility associated with such calculations. Dehler's et al. (4) have
shown that extrapolation of tumor volume, when extended far
beyond the tumor volume data, is inaccurate.

Chart 2 and Table 1 also show that 28G tumors responded to
castration to a significantly lesser degree than did 27G tumors,
while retaining the same growth rate and time to palpability when
grown in intact control rats. Moreover, 29G tumors showed
nearly identical growth and response kinetics, as compared to
28G tumors (data not shown). The difference in androgen
sensitivity between 27G and 28G tumors was due to a decrease in
the extent of response of 28G tumor cells to castration. In
summary, the progression to hormonal autonomy observed in
R3327-G tumors was first expressed as an increase in the time
to respond and then as a decrease in the extent of response.

**Effect of Androgen Supplementation on Growth Rate**

In general, the chronic supplementation of castrated R3327-G
tumor-bearing rats with TP caused tumor growth to approximate
the rate found in intact controls. Chart 3 shows that 22G tumors,
grown in castrates chronically supplemented (5 days/week) with
TP (0.625 mg/kg), and 27G tumors, grown in castrates supple-
mented with 10 mg/kg, grew slightly faster than did controls.

A bell curve-like relationship between TP concentration admin-
istered and growth rate was observed in 22G tumors. Supple-
mentation of castrates with low concentrations of TP (0.156 mg/
TUMOR PROGRESSION IN R3327-G TUMORS

Chart 3. Effect of androgen supplementation on the different transplant generations. The intact controls in A and B, respectively, were from the same experiment shown in Chart 2, A and B. A, intact; ○, castrates + TP (0.156 mg/kg); △, castrates plus TP (0.625 mg/kg); ◼, castrates + TP (2.5 mg/kg); ○, castrates + TP (10 mg/kg). In A, there were 9 animals/group, and in B, there were 7 animals/group.

kg) resulted in faster growth than unsupplemented castrates, approaching the growth rate of the intact controls. The peak tumor growth rate was found when TP (0.625 mg/kg) was administered: tumor growth was significantly faster than for the intact controls (Table 1). At still higher TP concentrations (2.5 and 10 mg/kg), the growth rate returned to, or was slightly lower than, that of the intact controls. These data show that, for the earlier generation tumors (22G), tumor growth rates below peak levels are observed when androgen is administered in high doses, as well as when androgen is deprived by castration. The later transplant generations (26-28G) were consistently stimulated by the highest concentration of TP (10 mg/kg) to grow at a faster rate than intact controls; 26-28G tumors were more resistant to inhibition from androgen excess. The differences in androgen sensitivity between early and late generation R3327-G tumors were observed to be more substantial in terms of sensitivity to androgen deprivation than to androgen excess.

The peak growth rate of R3327-G tumors is dependent on endogenous androgen levels. In earlier studies (18), we showed that serum testosterone becomes significantly reduced in tumor-bearing intact rats at large tumor volumes. It is unclear whether the reduction in serum testosterone in tumor-bearing rats is due to the effective removal of testosterone from the serum by the tumor and/or an indirect effect of the debilitation observed in these animals. The average growth curves in Chart 3A and the doubling times in Table 1 show that the growth rate of the intact control group decreased much more rapidly with increasing tumor volumes than that of the TP-supplemented groups. It is possible that, at the larger tumor volumes, endogenous testosterone levels in the intact controls became limiting, while in the TP-supplemented castrates, more constant serum testosterone levels were maintained.

Effect of Castration and Androgen Supplementation on Survival

The effects of androgen supplementation on survival (Chart 4) correlate with those on growth rate. Tumor-bearing castrates survived significantly longer than tumor-bearing intact controls or testosterone-supplemented castrates. 22G tumor-bearing castrates, castrates supplemented with TP, or intact controls survived significantly longer than did identically treated animals bearing 27G tumors.

Supplementation of 22G tumor-bearing castrates with TP (0.156 mg/kg) had no significant effect on average survival time (98.7 days) as compared to intact controls (101.3 days). Supplementation with 0.625, 2.5, or 10.0 mg of TP per kg, however, resulted in significantly shorter survival times (79.0, 60.5, and 80.0 days, respectively). Castrates treated with TP (2.5 mg/kg) had significantly shorter survival times than those treated with 0.625 or 10 mg/kg.

DISCUSSION

Two types of prostatic tumor progression have been evidenced in the Dunning R3327 lines: induced progression and spontaneous progression. Isaacs et al. (11) have shown that the well-differentiated R3327-H-Hopkins (Johns Hopkins) line is induced to progress to hormonal autonomy when tumors are
TUMOR PROGRESSION IN R3327-G TUMORS

grown in castrated rats. This transition to autonomy occurs in a single transplant generation as a consequence of the elimination of the androgen-dependent cell population. The androgen-independent cells show proliferation kinetics identical to the androgen-dependent population. The induction of progression to hormonal autonomy by castration occurs in the R3327-H-Hopkins line, however, does not occur in the poorly differentiated R3327-G line.

We have shown (18) that successive transplant generations of R3327-G tumors treated by castration respond to castration to approximately the same degree. These data suggest that the androgen-responsive cells in R3327-G tumors are not dependent on androgen and should be classified, instead, as being androgen sensitive (15, 16). Paradoxically, the growth rate response of R3327-G tumors to castration appears to be mediated by an increase in the number of cells exiting the cell cycle (i.e., cell death) (10). It appears that, while the rate of cell death is increased in R3327-G tumors grown in castrated rats, the androgen-sensitive stem cell line remains, and overall androgen sensitivity is not significantly affected. Compared to the R3327-H-Hopkins line, the R3327-G line is very resistant to the induction of progression by castration. High-dose diethylstilbestrol diphosphate treatment, however, does promote a significant progression of R3327-G tumors towards hormonal autonomy (18).

The results presented herein demonstrate that the R3327-G line undergoes spontaneous progression towards hormonal autonomy in later in vivo transplant generations. This means that R3327-G tumors maintained in intact male rats shifted towards hormonal autonomy without inducement (i.e., without being grown in castrated rats). The most significant changes in response to androgen ablation therapy were observed in the 22nd to 28th transplant generations. During this time, there were no obvious differences in histology (3, 18) or karyotype (3). Moreover, DNA content analysis by flow cytometry showed no significant changes in the DNA content of the aneuploid cell population (data not shown) (17). These findings differ somewhat from those reported by Isaacs et al. (12) concerning the R3327-H-Hopkins line and our own experience with the well-differentiated R3327-H-Miami (University of Miami) (3) line. Spontaneous progression in the H-lines most often involves an abrupt loss of sensitivity to androgen deprivation, accompanied by obvious alterations in histology, karyotype, and DNA content.

In prior studies with later-generation R3327-G tumors grown in intact rats (19), we found that these tumors contained high concentrations of cytoplasmic and nuclear androgen receptors. Recently, Diamond and Barrack (5) reported that R3327-G tumors contained significantly higher levels of cytoplasmic and nuclear receptors than did R3327-H tumors. Moreover, the R3327-AT-2 line (a fast-growing, androgen-independent, anaplastic tumor) contained undetectable levels of cytoplasmic and nuclear androgen receptors. The later-generation R3327-G tumors are unusual in that they show growth characteristics approaching those of the R3327-AT-2 line, yet they contain high levels of androgen receptors. These results indicate that the progression to hormonal autonomy in R3327-G tumors is not associated with a loss of androgen receptors.

The spontaneous reduction in the sensitivity of R3327-G tumors to androgen deprivation was found to be gradual and to occur in 2 stages. (a) With increasing numbers of transplant generations, more time was required for response to castration to be expressed by tumor volume (i.e., increased time to response). (b) In the later-transplant generations, the effect of castration was diminished in degree (i.e., decreased extent of response). The increase in time to response preceded the decrease in extent of response. The changes in the response characteristics of R3327-G tumors to castration did not always coincide with changes in the growth rate of these tumors when grown in intact rats. The growth rate in intact rats was stable between the 23rd and 29th generations, while response to castration was significantly reduced. These data suggest that the progressive changes in the characteristics of the response to castration and the growth rate in intact rats are independent (7).

The heterogeneity of human prostatic tumors is reflected in the different prostatic tumor animal models (3, 11). The R3327-G tumor model is most representative of the poorly differentiated, rapidly growing, androgen-sensitive tumors commonly found in Stage D disease. Our experience with human prostatic tumors from patients with later-stage disease shows that over 50% have an aneuploid cell population that is clearly identifiable by DNA content measurements (1). The relatively rapid growth of these tumors enhances the potential for mutation-selection and, hence, spontaneous tumor progression to hormonal autonomy. Our results with the R3327-G line indicate that the early treatment of later-stage prostatic cancers may reduce the potential for tumor progression, thereby increasing survival in some instances.

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