Beneficial Effects of Androgen-primed Chemotherapy in the Dunning R3327 G Model of Prostatic Cancer

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ABSTRACT

The objective of this study was to test the hypothesis that androgen administration prior to chemotherapy (androgen priming) may potentiate tumor cytotoxicity in hormone-responsive prostate cancer. Accordingly, six groups of Copenhagen rats bearing small (i.e., 40-mm3 median volume) Dunning R3327 G tumors were left untreated or received castration, chemotherapy, or a combination of the two, with or without androgen priming. Groups without priming included: intact untreated, castrate alone, intact plus chemotherapy, and castrate plus chemotherapy (cyclophosphamide, 30 mg/kg/day, for 2 days, with repeat cycle in 24 days) (Cx). To specifically evaluate the effect of androgen priming on Cx cytotoxicity, two additional castrated groups were studied. One received testosterone propionate (4 mg/kg/day) for 2 days prior to Cx and the other after Cx. Treatment effect was evaluated by quantitating tumor volume as well as animal survival to an ethically allowable, maximal tumor burden.

As expected, castration and Cx produced a retardation of tumor growth and prolongation of survival when compared to untreated animals. The addition of androgen priming prior to but not after Cx enhanced the degree of tumor suppression. Specifically, 26 days after the second Cx cycle, all androgen-primed tumors had regressed; 70% of tumors had disappeared and those remaining were barely palpable. At this same time point, tumors in all the other groups were actively growing and had volumes greater than initial values (P < 0.01). Although tumor regrowth occurred, median survival for the androgen-primed group was significantly prolonged, to 186 days versus 39 days (P < 0.01) for untreated animals and 153 days for the non-primed castrate plus Cx animals (P < 0.01). These data suggested that androgen priming potentiated the effects of Cx in castrate animals bearing R3327 G tumors.

INTRODUCTION

The benefit of chemotherapy to patients with advanced prostate cancer has been limited (1, 2). One potential explanation for this is the slow growth rate of prostate cancer (3, 4) and, consequently, its limited susceptibility to chemotherapy (5, 6). Extensive clinical and experimental efforts have attempted to enhance the chemosensitivity of slowly growing tumors by synchronization of tumor cell proliferation and recruitment of nonproliferating cells into the cell cycle (7–10). Under ideal conditions, the procedure utilized should only enhance tumor cell cytotoxicity, without affecting the normal host cells. Previous attempts which utilized chemical and physical methods have yielded limited results due to the nonspecificity of effects, with alterations of normal as well as cancer cell kinetics.

Hormonal manipulation provides a more specific means of altering cell proliferation. For example, androgen replacement following withdrawal elicits a significant increase in cell proliferation (11, 13) in the normal prostate gland. This response has also been observed in animal and human models of prostatic cancer (14, 15). Further, the initial favorable response of most patients to hormone ablation strongly suggests that a dependency on androgens for proliferative activity is retained after the neoplastic change (16).

Since manipulation of hormone levels can often be achieved with little attendant direct toxicity, a hypothesis has evolved that naturally occurring hormones can be utilized to specifically enhance tumor cell cytotoxicity. According to this theory, the hormone is first withdrawn and then, after a suitable period, readministered as a pulse. The objective is to induce a wave of tumor cell proliferative activity, which will thus render the cells more susceptible to precisely timed, subsequent chemotherapy.

Accordingly, we utilized the Dunning R3327 G rat model of prostate cancer to investigate the capacity of androgen withdrawal and replacement prior to chemotherapy to exert a potentiating effect.

MATERIALS AND METHODS

Animals. All animals used in this study were adult male Copenhagen rats obtained from The National Cancer Institute, Frederick Cancer Research Facility. Castration was via the scrotal route, using ether as the anesthetic agent. The animals were housed in an environmentally controlled room (12 h of light and 21°C) and were provided with food and water ad libitum, in the Department of Comparative Medicine, The Milton S. Hershey Medical Center of Pennsylvania State University. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animals Commission on Life Sciences. The animals were restricted to medicated water throughout the experimental period (17).

Tumor. The Dunning R3327 G tumor is characterized as being androgen responsive and morphologically poorly differentiated (18, 19). We originally obtained the Dunning R3327 G model in January 1985, through the generosity of Dr. Normal Block of the Department of Urology, University of Miami School of Medicine.

Tumor Inoculation Protocol. The donor Dunning R3327 G tumors selected for inoculation into the recipient experimental animals were approximately 1.5 cm in volume. Cells for inoculation were prepared using the method of Claflin et al. (20). Each experimental animal was restricted to medicated water throughout the experimental period (17).

Evaluation of Tumor Growth. Three dimensions of each tumor were serially measured using orbital calipers. From these measurements, tumor volumes were calculated using the formula (length × width × height) × 0.526 (21). The mean ± SE was calculated weekly for each experimental group. The data thus obtained are expressed in terms of the mean absolute tumor volume.

Circulating Testosterone Levels. Sera were obtained from intact, 7-day castrate, and androgen-repleted animals and were assayed for testosterone content using a previously described method (22). Androgen replacement consisted of two daily s.c. injections of testosterone (4 mg/kg) on days 7 and 8 after castration.

Evaluation of Androgen Depletion Followed by Repletion on Tumor Cell Proliferative Kinetics. Intact male Copenhagen rats were inoculated with 9 × 106 Dunning R3327 G tumor cells and left undisturbed until the tumor became palpable on day 14. Animals were then subjected to surgical castration. On day 7 after castration, testosterone replacement was initiated by daily s.c. injection (4 mg/kg) and continued for 7 days thereafter. Flow cytometry was utilized to quantify the percentage of...
cells in S phase for tumors under the following conditions: intact, castrate only, and after 1, 2, 3, and 7 days of testosterone replacement.

In order to quantify the percentage of cells in S phase, animals were given i.v. injections of 1.25 ml of bromodeoxyuridine (20 mg/ml of phosphate-buffered saline; Sigma), on the indicated days. Five animals, each bearing a single tumor, were evaluated under each condition. The animals were sacrificed 0.5 h later, and the tumor tissue was rapidly processed for flow cytometric analysis. The method used to process the tissue, as well as to detect bromodeoxyuridine-labeled nuclei, has been described in detail (23). In the present study, a directly fluoresceinated mouse monoclonal antibody was used (Becton Dickinson).

Flow cytometric data were collected using the Coulter Epics V instrument (Coulter Electronics, Hialeah FL) and analyzed using a previously described method (24).

Androgen Priming Protocol. Adult male Copenhagen rats, inoculated with 9 × 10^6 viable tumor cells and left until tumors were palpable, were randomized into the following experimental groups: CTCx, CCxT, CVCx, ICx, intact only, and castration only. All groups consisted of 16 animals each, with the exception of the ICx group, which was composed of 8. The period of castration for all animals which were subjected to androgens to potentiate chemotherapy are presented herein. The second experiment was performed to confirm the results obtained in the initial experiment. It was an exact repeat of the first, with the exception that a group of intact animals which received chemotherapy alone (ICx) was included. For purposes of clarity, because the two experiments were identical and the results were similar, the data were pooled. Unless otherwise stated, data are expressed as the mean ± SE. For flow cytometric data and serum testosterone levels, the significance of differences among treatment groups was determined by analysis of variance, using the Student-Newman-Keuls procedure to allow for multiple comparisons.

Tumor growth curves were fit using spline (piecewise differentiable) models with knots at the treatment times (25). Estimated spline curves account for random animal effects and time series autocorrelation, as described by Chi and Reinsel (26) and Diggle (27). We summarized growth by computing the mean log tumor volumes and their SE and covariances. These data were then used to characterize differences among the curves and to test for statistical significance of these differences. All growth curve computations were carried out in the S language (28).

Survival data were analyzed using a censored lognormal model. Survival times were considered to be censored for animals who died from the toxic effects of treatment. Such an analysis is appropriate if the distribution of times of death due to toxicity is independent of the distribution of times of death due to tumor morbidity, as seems plausible in these data. Computations for fitting the censored lognormal model were carried out in SAS Proc Lifereg (29).

RESULTS

Effect of Hormonal Manipulation on Tumor Cell Proliferative Kinetics. The percentage of cells in S phase under basal conditions (24.3 ± 2.1%) was high in the small tumors, as expected from the Gompertz mathematical description of tumor growth (Fig. 2). Castration caused a significant reduction in the percentage of S phase cells, to 14.6 ± 1.8%, and androgen replacement.

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2 The abbreviations used are: CTCx, androgen-primed chemotherapy; CCxT, chemotherapy before androgen priming; CVCx, castration plus vehicle plus chemotherapy; ICx, intact plus chemotherapy.
Castration only. These differences were not sufficient to suggest any form displayed diminished increases in body weight, compared to the intact untreated group and that which received chemotherapy (Cx) is to be given. Points expressed are means ± SE.

Circulating Testosterone Levels. In order to correlate changes in cell proliferative activity with androgen levels, serum testosterone was measured during the androgen withdrawal/replacement protocol (Fig. 3). After 7 days of castration, circulating levels of testosterone had fallen below the range of assay detectability (0.05 ng/ml). After testosterone replacement, testosterone levels increased at 24 and 48 h and declined thereafter. Days 3 and 4 were the times of planned cyclophosphamide administration. Circulating levels of testosterone, although lower than on days 1 and 2, remained above the physiological range until day 5.

Animal Weights. Animal weights were measured in order to assess the extent of toxicity to the surviving host animal, since deleterious effects on the host can secondarily affect tumor growth. Animal weight minus tumor weight was determined by assuming that the specific gravity of the tumor tissue was near unity.

Effect of Androgen-primed Chemotherapy on Animal Weight. Animal weights (Fig. 4) increased throughout the experimental period, although the groups which received chemotherapy in any form displayed diminished increases in body weight, compared to the intact untreated group and that which received castration only. These differences were not sufficient to suggest that tumor growth was significantly affected in an indirect fashion. Although the weight curve for the group which received androgen priming was almost identical to those for the other groups receiving chemotherapy, it was consistently on the lower side of these groups.

Tumor Growth. Fig. 5 is a log-linear plot of overall tumor growth. Compared to the intact group, castration alone slowed tumor growth, although as expected there was no absolute decline in actual tumor volume. All of the regimens that included chemotherapy led to decreases in tumor volume, although tumors eventually regrew in all surviving animals. Treatment of intact animals with cyclophosphamide was more effective than castration alone; for example, mean tumor volume on days 20 to 40 was lower by a factor of 4.2 in the ICx than in the castrate only group (P < 0.01). Tumor growth was always suppressed more in the three groups in which animals were both castrated and treated with cyclophosphamide than in the other groups. Among these groups, we measured tumor suppression by calculating, from our fitted cubic spline curves, the mean log tumor volume between days 40 and 60. Suppression was greatest in the androgen-primed (CTCx) group, where the mean log tumor volume in this range was roughly 4.1 (geometric mean tumor volume of 60.3 mm³). The next most potent tumor suppression was in the CVCx group, where mean tumor volume was greater than that in the CTCx group by a factor of 9.8. Mean log tumor volume in the CCxT group exceeded that in the CTCx group by a factor of 50. All three groups were significantly different at P < 0.01.

It should be noted that the only group to exhibit a sustained absolute decline in tumor volume was the group receiving androgen priming prior to chemotherapy (CTCx). The CTCx tumors began to decline at the initiation of androgen-primed chemotherapy and continued to do so until day 60. At this point, in only the CTCx group, 70% of the tumors disappeared. Two aspects should be specifically mentioned. First, tumor disappearance occurred 26 days after initiation of the second chemotherapy cycle and, second, these tumors subsequently
ceeded by tumor shrinkage, but this was accompanied by rapid cyclophosphamide administration. Death was generally preceded by weight loss and may have been a consequence of systemic toxicities rather than specific antitumor action. Such deaths reappeared and grew in a fashion similar to those that became very small but never completely disappeared on day 60.

Ethically Allowable Animal Survival. Using the censored log-normal model, median survival (see definition in “Materials and Methods”) in the CTCx group was found to exceed survival in any other group, in all cases with \( P < 0.001 \) (Fig. 6). Estimates of median survival and 95% confidence intervals for median survival are shown in Table 1. Survival with CTCx was estimated to be 186 days, with a 95% confidence interval of 176 to 198 days. The closest competitors were CVCx, with an estimated median survival of 153 days (95% confidence interval, 145 to 160 days), and CCxT, with an estimated median survival of 139 days (95% confidence interval, 132 to 147 days). The other regimens resulted in much shorter survival times.

The increase in median time of death from tumor morbidity achieved by androgen priming must be considered in light of the rate of death also observed in this group. Table 2 shows the number of animal deaths which occurred early in the study period, at times that were temporally related to the periods of cyclophosphamide administration. Death was generally preceded by tumor shrinkage, but this was accompanied by rapid weight loss and may have been a consequence of systemic toxicities rather than specific antitumor action. Such deaths typically occurred within 2 weeks of the end of the first or second cycle of chemotherapy. Hence, these deaths were considered to be directly attributable to chemotherapeutic toxicity. In the ICx group, 3 of 8 animals died of treatment toxicity, whereas 2 of 16 died in the CVCx group and 5 of 16 in the CCxT group, compared to 5 of 15 in the CTCx group. This suggests that chemotherapeutic toxicity was enhanced in any groups in which the animals had androgen present, either endogenous (ICx) or exogenously administered (CCxT and CTCx). Regardless, although this aspect should not be considered trivial, analysis of censored median survival revealed similar differences among the experimental groups (i.e., intact, 40 days; ICx, 101 days; castrate, 90 days; CVCx, 147 days; CCxT, 133.5 days; and CTCx, 183 days).

**DISCUSSION**

This report presents data obtained in a carefully controlled fashion which indicate enhancement of chemotherapeutic efficacy in animals subjected to androgen-primed chemotherapy. Specifically, 26 days after the second cycle, all tumors receiving androgen-primed chemotherapy had regressed; 70% were not detectable and those remaining were barely palpable. At this same time point, tumors in all other groups were actively growing and had volumes greater than at pretreatment. Ethically allowable animal survival for the androgen-primed group (censored as well as noncensored) was significantly prolonged.

Manipulation of tumor proliferative kinetics in order to potentiate chemotherapy is not a new idea. For many years, groups have applied various chemical, as well as physical, methods to enhance the chemosensitivity of tumors (7–10). The aim of these procedures is to increase tumor cell proliferative activity specifically at the period when the chemotherapeutic agent is administered. Consequently, because cytotoxicity is proportional to cellular proliferative activity, such procedures should result in a greater degree of tumor cell kill (5, 6).

In order for these approaches to be therapeutically efficient, the method utilized to manipulate cell proliferation should be specific for the tumor cell population. If it is not, the normal host cells might also respond and thereby also be destroyed to an enhanced degree along with the tumor cells. The use of hormones to manipulate tumor cell proliferative activity in endocrine-dependent tumors should obviate or significantly minimize the problem of enhanced host toxicity (30, 31).

The rationale of hormone-primed chemotherapy is that androgens, via mediation of proliferative activity and/or other aspects, may be used to directly enhance the effects of chemotherapy in prostatic tumor cells. A number of investigators have convincingly demonstrated that hormones stimulate mitotic activity in a partially synchronous manner, as well as possibly recruiting proliferatively quiescent cells into the cycle (32). Experimental data are available which specifically demonstrate androgenic stimulation of DNA synthesis and cell division in the normal rat prostate (11–13), animal prostatic tumors (14), human biopsies (33), and human tumor xenografts (15).

Several previous reports described the effects of androgen priming and chemotherapeutic efficacy. Grossman et al. (34) administered a priming dose of androgen 1 h prior to methotrexate in animals bearing Dunning R3327 G tumors. While androgen priming statistically potentiated the effects of chemotherapy, no absolute tumor regressions were observed nor was animal survival enhanced. The different timing of androgen administration could explain the more extensive responses obtained in our study. In the rat ventral prostate gland, the point of maximal proliferation is not achieved until 72 h after initia-

![Fig. 6. Ethically allowable animal survival. For each group, a censored log-normal curve (see "Materials and Methods") was superimposed onto the discretely decreasing observed data. Groups are identified by symbols described in Fig. 4.](image_url)
tion of testosterone replacement (11–13). In the tumor system, our data indicate a significant increase in S phase cells as early as day 1 after testosterone replacement. Consequently, although the tumor apparently responds more rapidly than the ventral prostate gland, the 1-h interval between androgen priming and chemotherapy may have been insufficient to induce significant numbers of cells into S phase at the time when maximal concentrations of methotrexate were present in the rat. Significantly, in evaluating their response, the authors suggested that the effect observed in the testosterone-primed group may be due to androgen-enhanced transport of methotrexate into the tumor cell (34).

In a somewhat similar experiment, Rosenberg et al. (35) also utilized a Dunning model. Cyclophosphamide was administered as a single dose (100 mg/kg) 3 days after a single priming dose of testosterone (5 mg/animal). Significant in this protocol, however, was the fact that the animals were not castrated and remained intact for the duration of the experiment (four courses). The results indicated that the tumor growth rate was equally reduced in the group receiving testosterone plus cyclophosphamide and the control groups (cyclophosphamide alone, orchietomy alone, and cyclophosphamide plus orchietomy). Animal survival was not significantly prolonged in the group receiving testosterone plus cyclophosphamide, although the group which received castration plus cyclophosphamide did exhibit enhanced survival. This experiment differs significantly from ours in two respects. First, the animal group which received androgen-primed chemotherapy was not castrated prior to treatment and, second, the model utilized was the well-differentiated, slowly growing Dunning R3327 PAP model (18). More recent data from our group indicated that the stimulative effects of androgen replacement following castration on the percentage of cells in S phase are temporally almost identical in this particular model and the rat ventral prostate gland (14) (i.e., the peak response of S phase cells occurs 72 h after testosterone replacement). In the case of the ventral prostate, however, the animals must be castrated beforehand.

Emerman and Siemiatkowski (36), utilizing the Shionogi androgen-responsive mouse mammary tumor, found that the combination of maintenance of submaximal levels of androgen and chemotherapy (cyclophosphamide, 100 mg/kg, plus Adriamycin, 6.5 mg/kg) resulted in a greater degree of tumor growth inhibition than either modality used alone. Unlike the Dunning R3327 G model, the Shionogi tumor exhibits distinct regression kinetics by priming or some other approach which may consequently involve.2 The tumors in this study were very small (palpable) at the initiation of treatment. Consideration of tumor size is an important aspect in therapeutic studies. Humphries and Isaacs (37) have clearly demonstrated that, when rats bearing established (1–2 cm³) R3327 G tumors were castrated and replaced with testosterone, no stimulation in the rate of DNA synthesis (cpm [3H]thymidine/h/cell) occurred. This was subsequently confirmed in our laboratory using [3H]thymidine autoradiography.3 Using other experimental approaches, these investigators further demonstrated that, in this particular tumor, androgens maintained tumor growth by inhibiting cell loss (37). When small (palpable) tumors were subjected to androgen withdrawal and replacement in the present study, however, an increase in nuclear bromodeoxyuridine incorporation was observed. Why small R3327 G tumors respond differently in this regard is not entirely clear. Since the R3327 G model is not induced, but instead propagated by serial passage, an age-related alteration in tumor cell androgen responsiveness is unlikely to be the explanation. Furthermore, utilizing several Dunning sublines, Henry and Isaacs (17, 38) have recently shown that there is an inverse relationship between animal tumor burden and response to cyclophosphamide alone or castration plus cyclophosphamide. Both of these aspects serve to emphasize that differences exist over the natural history of prostatic tumor establishment and growth and that favorable therapeutic responses are facilitated when treatment is begun as early as possible (17, 38, 39).

Overall, this report presents data obtained in a carefully controlled fashion which indicate that, when animals bearing the androgen-responsive Dunning R3327 G tumor are castrated and then subjected to androgen-primed chemotherapy, a distinct benefit will result. Clearly, because no tumors were completely eradicated, such a protocol is not currently optimal. The striking response should, however, encourage further effort toward protocol modification and addressing the unanswered questions, which may in turn result in therapeutic improvement via priming or some other approach which may consequently evolve.

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2 H. English, unpublished observations.
REFERENCES

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