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ANDROGEN-PRIMED CHEMOTHERAPY—EXPERIMENTAL CONFIRMATION OF EFFICACY

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Summary—A current hypothesis suggests that androgen administration prior to chemotherapy (androgen priming) may potentiate tumor cytotoxicity in prostate cancer. The Dunning R3327G rat prostatic tumor model was used to test this concept experimentally. Control groups without priming included (1) intact untreated, (2) castrate alone and (3) castrate + chemotherapy (cyclophosphamide, 30 mg/kg/day for 2 days with repeat cycle in 25 days—CTX). Two experimental groups received androgens, one before and one after chemotherapy. Treatment effect was monitored by quantitating tumor volume and animal survival.

Control groups receiving castration and chemotherapy had a retardation of tumor growth and a prolongation of survival when compared to untreated animals. Androgen priming before but not after chemotherapy enhanced the degree of tumor suppression. With the androgen-priming protocol, all androgen-primed tumors had regressed, 3/6 tumors had disappeared and 3 were only palpable. At the same time point, tumors in all the other groups were actively growing and had volumes greater than the initial values ($P < 0.01$). Median survival was significantly prolonged in primed animals 191 vs 40 days for untreated animals and 150 days for the nonprimed castration + chemotherapy animals ($P < 0.02$). These findings have been repeated with several replicate experiments. These observations confirm the hypothesis that androgen priming can potentiate chemotherapy in an experimental system.

INTRODUCTION

New treatment approaches for stage D_2 prostatic carcinoma are needed at the present time. This conclusion follows from reviewing results with the most promising of the recently introduced new treatment approaches, complete androgen blockade. Over the past 5 years, major investigative efforts have been directed toward the study of the concept of complete androgen blockade. Preliminary studies of this concept showed promise as a means of prolonging the survival of patients with metastatic prostate cancer [1]. However, controversy still exists as to whether this approach provides even marginal benefit, although several major trials have been conducted [2–5]. The largest study, a multicenter intergroup trial published in the *New England Journal of Medicine* [6], demonstrated that complete androgen blockade prolonged progression-free survival significantly, but only from 13.9 to 16.5 months ($P < 0.039$). Overall survival was prolonged by approx. 6 months to a median of 36 months. These data emphasize that, at best, complete and androgen blockade results in a median survival of patients of only 3 years. Stage D_2 prostate cancer must then be considered a highly aggressive disease with available treatments that are only temporarily effective. Numerous trials have evaluated the efficacy of chemotherapy in such patients. These have demonstrated only minimal beneficial results and no major prolongation of survival [7].

Recognizing the need to develop new treatment strategies, we have been evaluating an approach designed to enhance the potency of available chemotherapeutic agents [8]. This utilizes a hormone-depletion/-repletion strategy to recruit resting cells into the active cycle and to partially synchronize cells in cycle into the DNA synthesis or S-phase. It is known that cycling cells and those in S-phase are more sensitive to cytotoxic chemotherapy than cells that are in the G_0-(resting)phase. Our initial trial with this approach in men with prostate cancer relapsing after castration was negative and associated with significant toxicity [9]. For this reason, major efforts have been directed toward identification of key elements in this strategy in animal models. This report will review our experience with these animal studies.

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BIOLOGIC CONCEPTS

The natural history of prostatic carcinoma in patients suggests a gradual loss of hormone-dependence of cells within the tumor. By the time patients present with stage D_2 prostatic carcinoma, a certain fraction of cells remain hormone-responsive, whereas another fraction has developed hormone-independence. It should be recognized that the strategy of hormone-depletion/-repletion followed by chemotherapy does not directly influence the hormone-independent cell fraction. Consequently, our studies are directed toward identification of the events occurring in the hormone-responsive fraction.

PROGRAMMED CELL DEATH

Current concepts suggest that hormone-depletion results in programmed cell death of a subpopulation of cells which are absolutely *hormone-dependent*. Another population of cells remain quiescent within the tumor but are not actively destroyed during the period of hormone-depletion. These cells, termed *hormone-sensitive* cells, can be stimulated to regrow upon repletion of androgen.

The concept of programmed cell death has been studied in several models. We have evaluated this event in rat ventral prostate and in the PC-82 human prostatic tumor grown in nude mice [10, 11]. Abrupt depletion of androgen by castration results in a 95% reduction of epithelial cells within the rat ventral prostate gland [12]. A marked reduction in tumor volume occurs in the PC-82 tumor as a result of castration as well. This reduction in tumor volume results, at least partially, from active cell death. A morphometric method to study programmed cell death involves quantitation of the number of apoptotic bodies present. In the rat ventral prostate, there was a rapid increase in apoptotic bodies from $<1\%$ to nearly 5% occurring with a peak on day 2 after castration. The number of apoptotic bodies present rapidly diminished to nadir levels 5 days after castration. Comparative studies in PC-82 human tumors revealed a peak number of apoptotic bodies of approx. 3.5% on day 3, and a similar number present on day 9 with a reduction back to baseline by day 14 (Fig. 1). Studies in collaboration with Drs Kyprianou and Isaacs revealed that cell death in the rat ventral prostate is associated with a marked increase in calcium,

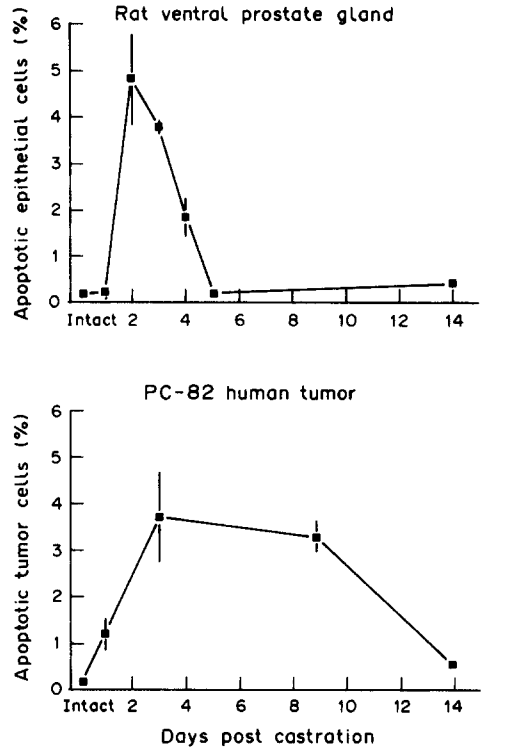

Fig. 1. Results from a morphometric method to quantitate the number of apoptotic bodies present on sequential days following castration. (Reprinted from English *et al.* [10]. Reprinted with permission of the publisher.)

magnesium-dependent, endonuclease. This enzyme increased from 10 to 35 U/gland, peaking on day 4 following castration. Similar changes occurred in the PC-82 tumor. Extraction of soluble DNA and electrophoretic analysis indicated that DNA is initially cleaved into specific nucleosomal-sized oligomers by day 1. Taken together, these indicate an active process of DNA degradation initiated by withdrawal of androgen. In other collaborative studies, these changes have been associated with an increase in TRPM-2, an enzyme activated by reductions in androgen [11]. While not necessarily causative for DNA breakdown, this gene marker provides correlative evidence of gene activation during androgen withdrawal.

ANDROGEN STIMULATION OF HORMONE-SENSITIVE CELLS

Even though the population of hormone-dependent cells dies during androgen withdrawal, an additional subpopulation (the hormone-sensitive cells) remains. These cells can be restimulated to grow upon initiation of androgen replacement. As previously reported, the number of glandular cells in rat ventral prostate

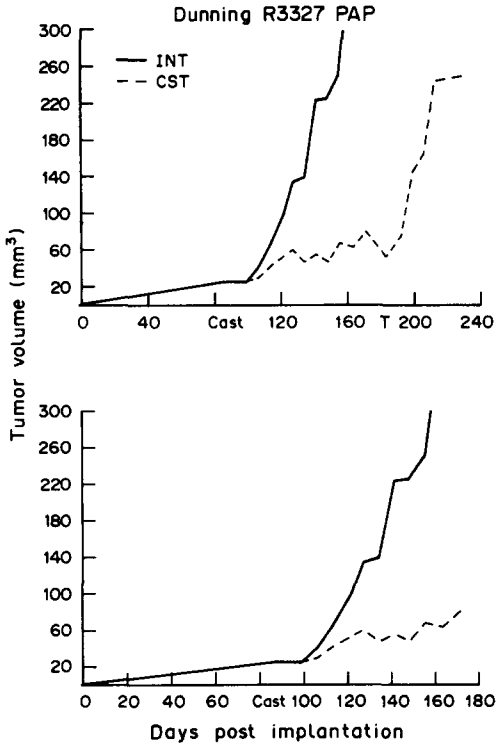

Fig. 2. Effect of castration and androgen readministration on the rate of rat prostatic tumor growth. INT = intact group; CST = castrate group; Cas = time of castration. *Top*: effect of castration followed by readministration of testosterone (T). *Bottom*: effect of long-term castration without testosterone readministration.

gland increased to near intact levels 11 days after restoration of normal serum testosterone concentrations [12]. A similar phenomenon can be demonstrated in the R3327 PAP prostate tumor model. As shown in Fig. 2 castration reduced the rate of tumor growth over that observed in intact animals. Reintroduction of androgen markedly increased the rate of tumor growth (Fig. 2) due to restimulation of persistent androgen-sensitive cells within the tumor.

PARTIAL SYNCHRONIZATION

Additional studies evaluated the degree of synchrony of hormone-sensitive cells upon repletion of androgen. Studied initially in rat ventral prostate, we and others demonstrated an increase in the percentage of [^{3}H]thymidine labeled cells from <1 to 10–30%, depending on cell type [12]. The epithelial, stromal, periacinar and endothelial cells all participated in this process. Partial synchronization also occurred in the PC-82 human prostate tumors subjected to 7 days of castration followed by testosterone repletion as assessed by BrDU flow cytometry. The percentage of S-phase of the total cells increased from $<1\%$ under castrate conditions to 4.5% upon administration of androgen. This increase could also be demonstrated by immunocytochemistry (not shown), a method which can precisely distinguish stromal from epithelial cell populations. With immunohistocytochemistry, an increase in the labeling index of epithelial cells from $<0.5\%$ under castrate conditions to approx. 10% upon androgen repletion was found. The stromal cell compartment does not appear to be altered by androgen depletion or repletion under these circumstances.

Rat models of prostatic carcinoma also exhibit partial synchronization during androgen repletion following androgen depletion. In well-differentiated Dunning R3327 PAP tumors, the [^{3}H]thymidine labeling index of epithelial cells is $<3\%$ under castrate conditions and increases to 7% on the third day following androgen repletion. Tumor size appears to influence the ability to demonstrate partial synchronization during androgen depletion or repletion. This is best shown in the less well-differentiated Dunning R3327G tumor. These tumors, when grown to 1.5 cm^3 in size, exhibit only a minimal reduction in S-phase from 18 to 15% upon castration and a nonstatistically significant rise to approx. 22%, 1–7 days after androgen repletion. In contrast, the R3327G tumors studied when just palpable exhibit more dramatic responses to androgen. Percentage S-phase levels in intact animals are approx. 25% and fall to 15% after 7 days of castration; 1–2 days following androgen repletion, the S-phase increases statistically significantly to 35%. Significant increases over basal persist for 3 days after androgen repletion.

POTENTIATION OF CHEMOTHERAPY BY ANDROGEN PRIMING

The Dunning R3327G tumor was selected as the best model in which to evaluate the effects of androgen depletion/repletion on chemotherapeutic potency. Tumors with a high percentage of cells in the S-phase would be expected to respond more effectively to chemotherapy than tumors with a smaller fraction. While the PC-82 tumor exhibited a maximum S-phase fraction of only 10%, the Dunning R3327G tumor could be induced by androgen repletion to an S-phase fraction of 35%. A detailed study protocol was designed to establish the beneficial effects of

androgen priming. The R3327G tumor was inoculated and tumors allowed to grow until just palpable. In the androgen-primed group, animals were castrated for 7 days, and then given testosterone propionate (4 mg/kg at 24-h intervals × 2) followed at 72 and 96 h by cyclophosphamide (30 mg/kg, CTC). Animals were then rested for 24 days and a second cycle repeated. Five groups of animals represented specific controls. These included: intact only, no other treatment; castrate only, no other treatment; intact plus chemotherapy (CVC); castration plus testosterone vehicle followed by chemotherapy (CCT); and castration followed by chemotherapy followd by testosterone. The characteristics of the testosterone plasma concentrations following and preceding testosterone injections were evaluated. Mean basal plasma testosterone concentrations approx. 2 ng/ml and fell to <0.1 ng/ml following castration. The two daily doses of testosterone propionate, 4 mg/kg, resulted in an initial rise of testosterone to 7 ng/ml after the first injection and peak levels of approx. 18 ng/ml 24 h after the second injection of testosterone propionate. Levels remained elevated above 3 ng/ml during the time of exposure to chemotherapy and then fell back to baseline.

The androgen-primed chemotherapy resulted in a greater inhibition of tumor volume than observed in all other treatment groups. Animal survival was also increased by the androgen-primed protocol. As shown in Table 1, results are best summarized utilizing definitions standard for evaluation of tumor responses in patients. Studies were conducted on two occasions utilizing groups of 8 rats each. As indicated, 50 and 80% of animals in the androgen-primed group were shown to have complete objective tumor regression and 50 and 20%, partial objective regression for a 100% rate of total objective regression in these two groups. Median survival was 190 and 172 days, respectively. In marked contrast to the results of androgen priming, animals receiving testosterone after chemotherapy experienced no complete or partial objective tumor regressions and survived for 140 and 84 days. These results highlight the efficacy of androgen-primed chemotherapy in producing a greater degree of objective tumor regression and animal survival than animals receiving the same agents but testosterone after the chemotherapy. An intermediate effect was observed when castration and chemotherapy were combined without the stimulatory effects of androgen. In these animals, no complete objective regressions were observed but 17 and 50% of animals, respectively, exhibited partial objective regression. Median survival in these animals were 161 and 154 days, respectively.

The androgen-primed chemotherapy clearly resulted in enhanced antitumor effects. Attribution of this result to cell kinetic mechanisms is only indirect and two possible sites of action of the potentiating effects must be considered. Firstly, the androgen-priming effects could be mediated at the level of the tumor; alternatively, systemic administration of androgen may have acted at the level of the host. Since cyclophosphamide requires activation by a metabolic step, it is possible that androgens could have affected this transformation at sites other than the tumor. To distinguish between these two possibilities, tumors were studied under similar conditions. Demonstration of similar potentiation of chemotherapy with androgen priming in *androgen-independent* tumors would suggest an effect at the level of the host. The Dunning R3327AT-2 and the Dunning R3327 PIF tumors were selected as androgen-independent analogs of R3327G. One of these tumors

Table 1. Summary of data: androgen-sensitive tumor Dunning R3327G

Experimental group		Complete objective regression (%)	Partial objective regression (%)	Total objective regression (%)	Median survival time (days)
Intact	I	0	0	0	44
	R	0	0	0	49
Intact and CTX	I	—	—	—	—
	R	0	20	20	110
Cast. only	I	0	0	0	90
	R	0	0	0	97
CVC	I	0	17	17	161
	R	0	50	50	154
CCT	I	0	0	0	140
	R	0	0	0	84
CTC	I	50	50	100	190
	R	80	20	100	172

I = initial experiment; R = repeated experiment.

(R3327AT-2) grows with a doubling time of 2 days in both the intact and castrate animals, whereas the Dunning R3327 PIF grows with a doubling time of 9 days in the intact animal and 10 days in the castrate. Thus, tumors spanning the rate of proliferation of the Dunning R3327G tumors (i.e. intact 4 days, castrate 8 days) were used for these studies. No enhancement of a therapeutic effect by androgen priming was observed in either the R3327AT-2 or PIF tumors (data not shown). These experiments suggest that androgen priming exerts its effects at the level of the tumor.

Even though active at the level of the tumor, androgen priming might exert its effects by mechanisms independent of the androgen receptor. To establish that the androgen receptor is necessary for the androgen potentiation effects, experiments were repeated utilizing the antiandrogen, flutamide. An identical protocol of androgen priming was utilized in animals bearing just palpable R3327G tumors. Prior to androgen administration, flutamide, 15 mg/kg, was administered daily as a means of blocking the effects of androgen at the level of the androgen receptor. Experimental groups included CTC (castration followed by testosterone followed by chemotherapy) alone, flutamide + CTC and flutamide + CVC (castration followed by vehicle followed by chemotherapy). Under these experimental conditions, CTC (androgen-primed group) potentiated the degree of tumor regression and flutamide blocked this priming effect.

KEY ELEMENTS IN ANDROGEN-PRIMED REGIMENS

In summary, the sequence of experiments demonstrate the validity of the concept that androgen priming can potentiate the effects of chemotherapy. These effects appear to occur at the level of the tumor and not of the host. Androgen priming appears to potentiate chemotherapy by an effect mediated through the androgen receptor as evidenced by the flutamide experiments. Several critical features of androgen-primed chemotherapy have been identified in our ongoing systematic studies. It is required to reduce serum concentrations of androgen to castrate levels for several days prior to androgen priming. The androgenic stimulation should be minimal and should not substantially exceed temporally the duration of chemotherapeutic action. Maximally effective chemotherapy is necessary to overcome the potentially adverse effects of androgen administration. Early initiation of treatment is required. The early nature of therapy is further supported by the studies of Isaacs *et al.* [13]. When treatment with castration + cytoxan was initiated in animals bearing tumors of 1–2 cm, animal survival was only 279 ± 23 days. Upon initiation of similar therapy with tumors <0.2 cm, survival, in contrast, was 405 ± 19 days.

UNANSWERED QUESTIONS

Our studies *in vivo* with experimental rodent tumors leave several questions unanswered. The role of adrenal androgen blockade in this experimental system is unclear. Rodents, as opposed to man, do not secrete a substantial amount of androgens from the adrenal. Consequently, this model cannot be utilized to test the contribution of adrenal androgens. The degree and duration of androgen stimulation has not yet been systematically tested. At the present time, it would appear reasonable that minimal androgen stimulation would reduce the potentially hazardous nature of the priming protocol. The precise timing of chemotherapy has also not been systematically addressed. Detailed examinations of these issues are ongoing.

CLINICAL TRIAL

Based upon these animal experiments, we have initiated a new trial in patients. Our previous studies examined the concept of androgen priming in men relapsing following surgical castration. Such patients exhibit large tumor burdens and only a small fraction of cells remain androgen-sensitive. The new trial was designed to incorporate several of the principles identified in the animal studies. The major element included initiation of treatment earlier in the disease and at a time of minimal tumor burden. Consequently, men with stage D_2 prostate cancer are now entered into the study prior to castration. Immediately following orchiectomy, patients are started on hydrocortisone, 20 mg twice daily in order to inhibit the secretion of adrenal androgens and followed for 3 months. Men experiencing objective tumor progression at the end of 3 months are excluded from study. Those experiencing objective regression or stabilization begin the protocol of androgen-primed chemotherapy. Each subject receives cytoxan, adriamycin and 5-fluorouracil

preceded by either placebo or halotestin, 5 mg twice daily. Cycles continue at 3-weekly intervals over a 2-year period. Forty-eight patients have now been entered into this trial. In contrast to our previous study, we have observed no patients with spinal cord compression induced by androgen priming and only minimal increases in bone pain have been observed. Consequently, it would appear that androgen priming in the presence of minimal tumor burden is safer than androgen priming in the presence of extensive disease. The study is not yet sufficiently mature to compare objective response or survival data.

POTENTIAL PITFALLS IN ONGOING CLINICAL TRIALS

The animal studies presented identified several requirements for successful androgen-primed chemotherapy. Thoughtful analysis reveals that several major problems remain. It is likely that human prostate tumors are markedly heterogeneous with respect to individual cell doubling time, hormone-dependence, hormone-sensitivity and other biologic characteristics. Assessment of the degree of synchronization of tumor cells after androgen administration in patients requires sequential biopsies. We have found this to be difficult on a practical level. The principle of androgen-primed chemotherapy represents a strategy which can be potentially harmful if the stimulatory effects of androgen are not exceeded by the enhanced chemotherapeutic potency induced. Trials studying these concepts must, therefore, be considered highly experimental and conducted under stringent conditions. Further study of the mechanisms inducing synchronized cell proliferation are required before priming protocols can be optimized. Animal models provide an appropriate means to establish the various principles involved in therapeutic efficacy of these approaches.

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