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Letter to the Editor

Effects of Testosterone and Dihydrotestosterone on Malignant Transformation in C3H10T½ Cells*

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THE ROLE of androgens in the etiology of human neoplasia is poorly understood. The involvement of these hormones in the progression of human prostatic cancer is suggested by the effectiveness of androgen ablation procedures [1–3] and of anti-androgen treatment [4] in inducing tumor regression, although a causative role for androgens in either benign prostatic hypertrophy or prostatic carcinoma has not been established. Animal experiments have not succeeded in producing prostatic carcinoma through androgen administration [5], with the exception of one report of prostatic adenocarcinoma produced in the Nb stain of rats following prolonged treatment with testosterone propionate pellets [6].

While androgens appear to have a permissive effect on the growth and development of many prostatic tumors, they exhibit a regressive effect in the treatment of many animal [7] and human [8, 9] mammary tumors. As the tumors affected are predominantly estrogen receptor-positive [10], and as estrogens are frequently permissive agents for growth of such tumors [9], an antagonistic relation between androgens and estrogens at the receptor level has been inferred [11, 12]. That such a relationship exists has been strengthened by the finding of reduced cytosolic estrogen receptor levels in regressing mammary tumors following androgen therapy [13].

A possible role of androgens in the etiology of breast cancer has not been established, however, as contrasted with experiments demonstrating the ability of estrogens to induce mammary [14] and other tumors [14] in animals following prolonged hormone administration. Testosterone has, however, been reported to cause inhibition of acetylaminofluorene-induced mammary tumorigenesis in rats [15], and other reports have suggested that androgens reduce the incidence of spontaneously occurring leukemia in specific strains of mice [16, 17] and counteract the effects of estrogens in the induction of murine lymphoma [18].

Given the considerable uncertainty concerning the role of androgens in the process of tumorigenesis, as well as the conflicting effects of these hormones on tumor growth in the few animal and human systems in which they have been studied, we have investigated the effects of the androgens, testosterone and dihydrotestosterone, on X-ray induced malignant transformation in tissue culture. We have utilized the C3H10T½ cell line, in which we have previously reported enhancement of transformation induced by the estrogen 17-β-estradiol (both alone and in combination with X-irradiation) [19], and by the glucocorticoid hormone, cortisone [20].

The C3H10T½ transformation assay was utilized for the experiments reported here; this system has been used extensively for the modification of carcinogen induced transformation *in vitro*, as has been recently reviewed [21]. We have previously reported the interactions of several hormones with radiation in the induction of transformation *in vitro*: details of our experimental techniques for radiation transformation experiments using 10T½ cells have been described elsewhere [19, 20, 22]. Stock cultures were maintained in 60 mm Petri dishes and

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were passed by subculturing at a 1 : 20 dilution every 7 days. The cells used were in passages 9–14. They were grown in a humidified 5% CO_2 atmosphere at 37° C in Eagle's basal medium supplemented with 10% heat-inactivated fetal calf serum and gentamycin. For the transformation assays, the experimental design involved seeding a sufficient number of cells such that there would be approx. 300 viable (surviving) cells in each dish of each treatment group. As the plating efficiency of C3H10T½ cells varied with the particular serum lot being utilized at the time of each experiment, different numbers of cells were initially seeded to account for the varying plating efficiencies. Cultures to be irradiated received more cells than control cultures to compensate for the cell killing effect of the radiation exposure.

To determine the number of viable cells actually present in each dish and exposed to the various experimental treatments, each of the cell suspensions to be utilized for a transformation assay was diluted and seeded at low density [to result in approximately 80–100 surviving cells (colonies) per 100 mm Petri dish] so that the number of viable cells could be directly determined in each treatment group. These cultures (three dishes per treatment group) were exposed to the experimental treatments in the same manner as the higher cell density cultures which were utilized for the transformation assays, and were then utilized to determine the plating efficiency for each experimental treatment group. These dishes were terminated after 10 days of incubation; plating efficiencies were determined from the number of viable cells (represented as colonies of cells containing at least 50 cells each at 10 days post-irradiation) and the number of cells initially seeded in each group. It is assumed that the plating efficiency as determined in these relatively low cell density cultures directly applies to the higher cell density cultures utilized for the transformation assays; the number of viable cells per dish in the dishes utilized for the transformation assays is determined from the plating efficiency figures and the number of cells initially seeded for the transformation assay in each treatment group. Cultures utilized for the transformation assays were maintained for 6 weeks, with the cultures reaching confluence at approx. day 10 of the transformation assay period. After the cultures reached confluence, the serum concentrations were reduced to 5% and maintained at that concentration throughout the remainder of the transformation assay period. At 6 weeks, the experiments were terminated and the cultures were fixed, stained and scored for the presence of transformed foci. The morphology of the transformed cells and their corresponding tumorigenicity are approximately the same for either chemically [23, 24] or radiation [25] induced transformants. As has previously been described in detail [23–25], both Types 2 and Type 3 transformed foci appear as large, densely packed and extensively piled up masses of cells overlying the confluent monolayer of normal C3H10T½ cells, with Type 3 foci in addition exhibiting marked criss-crossing and swirling of highly stellate cells at the border of the focus. The cells of both Types 2 and 3 foci are highly basophilic and show marked increases in the nuclear/cytoplasmic ratio. Both Types 2 and 3 foci were scored as transformants in our studies as both of these types of transformed foci contain cells which are highly tumorigenic when injected into animals. When cloned and injected into animals, Type 3 cells are tumorigenic in 80–100% of inoculated mice, while Type 2 cells are tumorigenic in 60–75% of inoculated mice [25].

Testosterone and dihydrotestosterone were purchased from Sigma, and were each used at concentrations of 0.1 μg/ml. To determine whether any agent can affect radiation induced transformation, we routinely utilize the highest nontoxic concentration of the agent in C3H10T½ cells (as determined in preliminary toxicity studies). Thus, even though dihydrotestosterone is 2–3 times more androgenic than testosterone in animal studies, we studied the same concentration of these hormones in our *in vitro* studies as their cytotoxicity to C3H10T½ cells was similar. Both hormones were added to cultures immediately after the radiation exposure, and treatments were continued at once per week intervals throughout the transformation assay period (hormones were added at the regular scheduled media changes).

Three separate experiments were performed to determine the effects of testosterone and its primary metabolite, dihydrotestosterone [26], on radiation-induced transformation. The results of these experiments are shown in Table 1. All three experiments showed the same trends for the data gathered: both testosterone and dihydrotestosterone significantly suppressed radiation induced transformation *in vitro*. As some investigators may wish to calculate the transformation frequency per surviving cell for each treatment group, we have included the data for the number of viable cells exposed to the various treatments so that such calculations can be performed. We have compared the results obtained for the transformation yield in various treatment groups in our studies in terms of the fraction of dishes containing transformants in each group, with a Chi-square analysis performed to determine whether the observed differences are statistically significant. This is a widely accepted method to determine statistically significant differences in yields of transformed foci in C3H10T½ cell cultures [27].

Table 1. *Effects of testosterone and dihydrotestosterone on radiation transformation* in vitro

Treatment group	Experiment No.	Number of cells initially seeded per dish (in the transformation assays)	Plating Efficiency (%)	Number of viable cells per dish (in the transformation assays)	Total number of viable cells (in all dishes for each experiment)	Total number of foci observed (Types 2 + 3)	Fraction of dishes containing transformed foci (Types 2 + 3) Per experiment	Total*
1. Controls—	1	900	65.0	585	5265	0	0/9	
no	2	1000	21.3	213	2343	0	0/11	0/30
treatment	3	900	33.9	305	3050	0	0/10	
2. 400 rads	1	6000	10.7	642	15408	6	5/24=0.21	
	2	10,000	2.5	250	5250	6	5/21=0.24	16/69=0.23
	3	10,000	5.1	510	12240	6	6/24=0.25	
3. 400 rads +	1	6000	11.6	696	16704	2	2/24=0.08	
testosterone	2	10,000	2.2	220	4620	1	1/21=0.05	4/69=0.06
(0.1 μg/ml)	3	10,000	4.7	470	11280	1	1/24=0.04	
4. 400 rads +	1	6000	11.1	666	17982	1	1/27=0.04	
dihydrotesto-	2	10,000	3.2	320	6720	2	1/21=0.05	2/48=0.04
sterone (0.1 μg/ml)								

*Statistical analysis (Chi-square; pooled results of the three separate experiments shown): Groups 2 vs 3 or 4, $P < 0.01$.

Either method by which our results are analyzed leads to the conclusion that testosterone and dihydrotestosterone suppress radiation transformation *in vitro*.

The contrast between the transformation inhibitory effects of these androgens *in vitro* and the transformation enhancing effects found for the estrogen 17β-estradiol *in vitro* in the same cell system [19], are compatible with the hypothesis that prostatic hyperplasia may result from a hormonal imbalance in which a decrease in androgen levels results in an increased estrogen/androgen ratio [28]. Thus, androgens may compete with estrogens in the normal prostate and exert a "protective" effect towards hyperplasia. As prostatic cells are known to contain both androgen receptors and estrogen receptors [28], it is not clear whether any antagonism between these classes of hormones reflects competition for a common receptor or conflicting effects resulting from binding of each class of hormone to its own receptor. Other possible anti-tumorigenic effects of androgens, in inhibiting formation of chemically-induced mammary tumors and formation of spontaneously arising as well as estrogen-induced tumors of the hemapoietic system, have been referred to above [15–18].

The transformation-suppressive effects of androgens reported here may be related to the inhibitory effects of androgens on growth of estrogen-dependent mammary tumors [7–9], although it is not clear that androgens operate by the same mechanisms in tumors and in normal tissues. In this regard, it is of interest that the synthetic androgen methyltrienolone has recently been reported to inhibit growth of estrogen receptor-containing endometrial carcinoma cells in culture [29]. Androgens may thus antagonize one or more receptor-related processes through which estrogens enhance tumor growth.

The present results demonstrating androgen inhibition of X-ray transformation *in vitro*, together with our previous results showing estrogen enhancement of *in vitro* transformation in combination with X-irradiation or alone [19], are thus consistent with the available *in vivo* data in which androgens are generally found to be inhibitory to tumor development while estrogens are found to be stimulatory. The C3H10T½ cell line may thus represent a useful *in vitro* system in which to study the apparently antagonistic effects of these two groups of hormones.

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