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Growth-stimulating effect of adrenal androgens on the R3327 Dunning prostatic carcinoma

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Summary. Adrenal androgens are discussed as a reason for tumor progression after androgen ablation therapy. Because of the difference in the secretion of androgens by the adrenals of humans and rats, there is no reliable tumor model to study the role of adrenal androgens in tumor progression. Therefore, the main adrenal androgens were administered to rats in order to mimic human endocrine conditions. Application of dehydroepiandrosteron-sulfate (DHEA-S) alone or a mixture of androstendione (A), 11β-hydroxyandrostendione (OHA), dehydroepiandrosterone (DHEA), and its sulfate (DHEA-S) to castrated rats caused only a slight increase of prostate and seminal vesicle weights. Contrary to these findings, growth of the R3327 prostatic carcinoma in castrated rats was greatly stimulated by these adrenal androgens up to the level of the intact control. Thus, in spite of androgen ablation, tumor progression could be induced by exogenous adrenal androgens.

Key words: R3327 prostatic carcinoma – Adrenal androgens – Relapse

Endocrine therapy of disseminated prostatic carcinoma is managed by various established methods [e.g., orchidectomy, estrogens, antiandrogens, or luteinizing hormone releasing hormone (LHRH) agonists]. The therapeutic effect is mainly due to the marked inhibition (90%–95%) of androgens of testicular origin. About 5%–10% of the circulating androgens are of adrenal origin [17]. The secretion of adrenal androgens is not affected by orchidectomy, estrogens, or LHRH application. Antiandrogens, however, are able to block the binding of testicular and adrenal androgens to androgen receptors [5].

The role of adrenal androgens in stimulating tumor progression after successful endocrine therapy is discussed controversially. There are two main explanations for this relapse that have been covered in the literature: (a) growth-stimulatory effect of the remaining adrenal androgens [16] and (b) growth due to hormone-independent cell clones [11].

Direct secretion of testosterone (T) by the adrenals is not affected by orchidectomy [21]. Suprisingly, the intraprostatic concentration of the potent androgen dihydrotestosterone (DHT) is reduced only about 55% after castration [8]. Another hint for the growth-stimulating effects of adrenal androgens is the clinical response to chemical or surgical adrenalectomy of some patients refractory to orchidectomy [4].

The main androgen precursors secreted by the human adrenals are androstendione (A), dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEA-S) [7]. These compounds are only weak androgens [20] but can be converted by human prostatic tissue to the potent androgens T and DHT [9].

In contrast to human serum which contains mainly T, DHEA, and 17α-hydroxypregnenolone, rat serum contains T and progesterone (P) as dominant steroids [19]. Therefore, rat prostatic tumor models could not be used for investigations of the role adrenal androgens in the human relapse phenomenon [2]. A model using the R3327 rat prostatic carcinoma stimulated by the main human adrenal androgens [A, DHEA, DHEA-S, and 11β-hydroxyandrostendione (OHA)] is the topic of this paper.

Material and methods

Growth stimulating effect on accessory sex organ weights

Mature male SD rats (220 g at beginning of the test, 5–6 rats/group, from Ivanovas, Kissleg, FRG) were used. Animals were castrated via the scrotal route under ether anesthesia 5 days before the start of the experiment. Intact and castrated rats were injected with the test compounds for 14 consecutive days. Twenty-four hours after the last injection prostates and seminal vesicles were removed, dissected free from adhering fat and tissue, blotted dry (prostates) or dried overnight at 100°C (seminal vesicles), and weighed.

Table 1. Effect of DHEA-S on accessory sex organ weights of castrated rats

	Dose[a] (mg)	Prostate[b] (mg)	Seminal vesicle[c] (mg)
Control		479 ± 102	116 ± 15
Castration		83 ± 15	19 ± 5
DHEA-S	11.2	74 ± 31	19 ± 3
	16.8	99 ± 22*	18 ± 2
	22.5	49 ± 31*	20 ± 3

[a] Application 3× a week olive oil
[b] Average, dry weight
[c] Average, wet weight
* Significant ($P < 0.01$) compared with castration

Hormone-dependent R3327 prostatic carcinoma

R3327 prostatic tumors and male Copenhagen rats were kindly provided by Dr. N. Altman, Papanicolau Cancer Research Institute, Miami, Fla, USA. F_1 hybrids of Copenhagen und Fisher rats (Charles River Wiga, Sulzfeld, FRG) were bred in the authors' laboratories. Tumors (2–3 mm^3 pieces) were implanted s.c. into 9–10-week-old rats (2 tumors/rat) [22, 23]. The treatment was started 96 days after implantation, the animals being distributed into groups of 7–8 rats, with an average tumor area of 19.6 + 1.1 mm^2/tumor. Tumor areas were determined weekly by calculating the product of caliper measurements made in two perpendicular diameters. At the end of therapy blood was taken via cardiac puncture under ether anesthesia. Tumors, seminal vesicles, prostates, and testes were removed, dissected free from adhering tissue, and weighed; seminal vesicles were dried overnight (100°C) before weighing.

Histology and morphometry

Tumor tissue was fixed in 7% phosphate-buffered formalin and routinely embedded in paraffin. For histologic examination and morphometrical analysis, 3-μm-thick sections were stained with hematoxylin and eosin. Morphometry of the tumors was performed nonautomatically on a Reichert projection microscope at 260× magnification. Volume densities of the tumor epithelium and the tumor stroma were determined using the point counting method with a 42-point Weibel grid [26]. Hits epithelium (including hits lumen) and hits stroma were counted.

Determination of testosterone and dihydrotestosterone in plasma

Testosterone was determined by a T-specific, ^{125}I-labelled radioimmunoassay (Biodata Testosterone Maia, Serono, FRG) according to the recommendations of the supplier. For the determination of DHT in plasma, 1.5 ml of plasma were extracted twice with diethylether. The extracts were combined and evaporated to dryness. Afterwards DHT was separated from T by chromatography on a celite column [1]. To determine the recovery rate, a ^{3}H-DHT standard was added to the sample before chromatography. The DHT fraction was determined by tritiated radioimmunoassay (5α-DHT Kit, Radioassay Systems Laboratories, USA). Samples with recoveries below 40% were eliminated.

Determination of testosterone and dihydrotestosterone in tissue

To determine T and DHT in tumor tissue 2–3 g tissue samples from each group were minced using a pair of scissors and homogenized in 10 ml ethanol with a mixing rod (Ultra Turax). The homogenized samples were then extracted with an equal volume of diethylether overnight. After centrifugation, the ether phase was collected, and the pellet was extracted a second time with 5 ml of ether. The combined ether extracts were evaporated to dryness, dissolved in methanol, extracted with hexane to eliminate fat, and evaporated again. Then ^{3}H-T and ^{3}H-DHT standards for recovery determination were added and the samples chromatographed as described above to separate T and DHT. The T and DHT fractions were evaporated, and levels of the two steroids were determined by tritiated radioimmunoassay (5α-DHT kit from Radioassay Systems Laboratories and T kit from Wien Laboratories, USA). Samples with recoveries below 40% were eliminated.

Statistics

The significance of the difference between two means was evaluated on the basis of the U test according to Wilcoxon, Mann, and Whitney.

Results

Effect of adrenal androgens on accessory sex organ weights of castrated rats

The difference in plasma concentrations of adrenal androgens in humans and in rats [1], especially of DHEA-S, was the reason for evaluating the effect of DHEA-S on the accessory organ weights of castrated rats at a dose which gave a similar plasma concentrations to those reported in humans (2,400 μg/24 h) [18].

In contrast to the work by Moguilewski et al. [18] we did not use osmotic minipumps but administered DHEA-S dissolved in olive oil subcutaneously three times a week at doses of 22.5, 16.8, and 11.2 mg/week per rat. Only at a dose of 16.8 mg/week per rat could a slight but significant increase in the weight of prostates (19% of intact control) be observed (Table 1). There was no effect on seminal vesicle weights at all.

One possible explanation for the disagreement of these results with those published by Moguilewski et al. [18] can be the different modes of application. For this reason, we repeated the experiment, including one group with a daily application of DHEA-S, another group which daily received the same adrenal androgen mixture (A, 11β-OHA, DHEA, DHEA-S) as that used by Moguilewski et al. [18] (Table 2), and a third group that received the adrenal androgen mixture three times a week. In this experiment DHEA-S caused only a very slight stimulation of accessory sex organ weights, even when administered daily.

Using a mixture of the main human adrenal androgens (A, 11β-OHA, DHEA, DHEA-S) as described by Moguilewski et al. [18], a weak but significant increase of prostate weights was only found in the group which received the adrenal androgen mixture daily. However,

Table 2. Effect of DHEA-S and adrenal androgens on accessory sex organ weights of castrated rats

	Applications per week	Prostate[c] (mg)	Seminal vesicle[d] (mg)
Control		369 ± 77	127 ± 50
Castration		23 ± 4	17 ± 2
DHEA-S[a]	3	35 ± 8*	20 ± 3
DHEA-S[a]	7	31 ± 11	21 ± 3
Adrenal androgens[b]	3	29 ± 5	20 ± 3
Adrenal androgens[b]	7	42 ± 5*	22 ± 3

[a] Dose: 16.6 mg/week in olive oil
[b] Dose: androstendione 0.76 mg/week, 11β-hydroxyandrostendione 0.42 mg/week, DHEA 1.00 mg/week, and DHEA-S 16.8 mg/week in olive oil
[c] Average, dry weight
[d] Average, wet weight
* Significant ($P < 0.01$) compared with castration

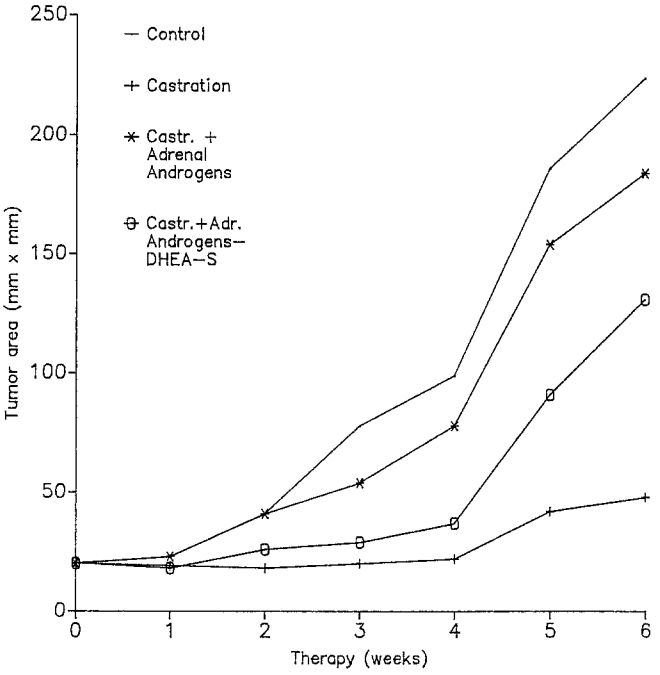

Fig. 1. Effect of exogenous adrenal androgens on the growth of the R3327 prostatic carcinoma of the rat. Adrenal androgens (per rat, androstendione 0.76 mg/week, 11β-hydroxyandrostendione 0.42 mg/week, dehydroepiandrosterone 1.00 mg/week, and its sulfate 16.8 mg/week) were administered daily, starting at the time when the tumors reached an average area of 20 mm^2/tumor

these weights were far below the values of the intact control and were much less than the about fourfold increase reported by Moguilewski et al. [18].

Effect of adrenal androgens on R3327 prostatic tumor

As differences in the response of normal and malignant prostatic tissue to androgens have been reported [25], we tested the effect of the adrenal androgen mixture on the growth of the hormone-dependent R3327 Dunning prostatic carcinoma of the rat (originally obtained from Dr. Altman) in castrated rats. Castrated male Copenhagen-Fischer F1 hybrids with established tumors were used. Application of adrenal androgens was done daily as described above. In contrast to the accessory sex organ weights of the normal rat, after 6 weeks of treatment the tumors showed a dramatic increase in growth. Growth curves of intact controls and of castrated, adrenal androgen-treated animals were almost identical (Fig. 1), whereas castration alone caused a strong decrease of tumor growth. Omitting DHEA-S in the adrenal androgen mixture led to a pronounced difference on tumor growth; stimulation was only 59% of that of intact controls at the end of the experiment.

Tumor weights of adrenal androgen-treated rats were compared with the intact control group; the group without DHEA-S had only 59% of the tumor weight of the intact control group, whereas supplementation with the entire adrenal androgen mixture caused a increase in tumor weight to 92% of that of the intact control (Table 3).

In the same animals in sharp contrast to the prostatic tumors, even after 6 weeks of therapy the weights of the accessory sex organs were only slightly increased. Seminal vesicles showed less than twofold increase in weight compared with castration. Moreover, prostates of animals treated with adrenal androgens or adrenal androgens

Table 3. Growth-stimulating effect of adrenal androgens on the R3327 prostatic carcinoma of the rat after 6 weeks of therapy

	Tumor area		Tumor weight		Seminal vesicle (mg)	Prostate (mg)
	(mm^2)	T/C (%)	mg ± SE	T/C (%)		
Control	224	100	2,383 ± 1,584	100	199	317
Castration	48	22	550 ± 450	23	25	58
Castration + adrenal androgens	184	82*	2,192 ± 1,599	92*	40*	69
Castration + adrenal androgens-DHEA-S	131	59*	1,190 ± 1,058	50*	37*	69

* Significant ($P < 0.01$) compared with castration

Table 4. Growth-stimulating effect of adrenal androgens on the R3327 prostatic carcinoma of the rat after 6 weeks of therapy

	Tumor area		Tumor weight		Seminal vesicle (mg)	Prostate (mg)
	(mm^2)	T/C (%)	(mg) ± SE	T/C (%)		
Control	366	100	6,719 ± 5,196	100	154	291
Castration	113	31	1,796 ± 1,948	27	27	43
Castration + adrenal androgens	378	103*	6,151 ± 7,451	92**	76*	50*

* Significant ($P < 0.01$) compared with castration
** Significant ($P < 0.05$) compared with castration

Table 5. Effect of adrenal androgens on plasma and tissue levels of dihydrotestosterone (DHT) and testosterone (T) after 6 weeks of therapy

	DHT plasma level		T plasma level		DHT tissue level		T tissue level	
	(ng/ml ± SD)	*n*	(ng/ml ± SD)	*n*	(ng/ml ± SD)	*n*	(ng/ml ± SD)	*n*
Control	0.11 ± 0.05	4	0.31 ± 0.10	8	1.10 ± 0.69	6	0.34 ± 0.29	4
Castration	0.14 ± 0.08	2	0.04 ± 0.05	8	0.04 ± 0.02	2	0.00 ± 0.00	2
Castration + adrenal androgens	0.10 ± 0.05	3	0.19 ± 0.06	6	0.73 ± 0.34	8	0.19 ± 0.32	8
Castration + adrenal androgens-DHEA-S	0.11 ± 0.04	4	0.23 + 0.10	8	0.68 ± 0.14	6	0.28 ± 0.23	6

Table 6. Morphometric determination of stroma and epithelium after administering adrenal androgens for 6 weeks

	Volume density (%)	
	Epithelium	Stroma
Control	59	41
Castration	43	57
Castration + adrenal androgens	50	50
Castration + adrenal androgens-DHEA-S	46	54

Average of four determinations

without DHEA-S had almost the same weights as those of castrated (Table 4).

This experiment clearly showed that the tumor-inhibiting effect of castration could be overcome by application of adrenal androgens. On the other hand, accessory sex organs were almost unresponsive to the applied adrenal androgens. The tumor growth-stimulating effect of the adrenal androgens was fully confirmed in an additional experiment on the R3327 prostatic carcinoma of the rat as shown in Table 4.

Determination of testosterone and dihydrotestosterone levels in plasma and tissue

In order to confirm that the increase in tumor growth is really due to an enhanced level of plasma and tissue androgens, T as well as DHT were measured. A five- to sixfold increase of plasma T could be observed at the end of the experiment (Table 3, Fig. 1). The presence of DHEA-S did not further stimulate plasma T levels. In comparison with intact controls, DHT plasma levels were not elevated after therapy with adrenal androgens with or without DHEA-S (Table 5).

The fact that castration did not affect T and DHT levels in plasma and tissue to the same extent induced us to determine the T and DHT concentrations in tumor tissue. In contrast to plasma levels, the DHT level in the tumor was strongly elevated, as was the case for the T concentration (Table 5). In comparision with castration, an almost 20-fold increase was observed in tumor tissue. Omitting DHEA-S did not affect the tissue levels of T and DHT (Table 5). Because of the increased plasma T levels, the main portion of the administered adrenal androgen seemed to be converted to T in the peripheral tissue of the rat but not directly within the tumor.

Histology and morphometry

To evaluate the cellular reactions caused by castration and application of adrenal androgens, histological examination of the tumors was performed. The histological pattern of the tumors of the control group was similar to well-differentiated human adenocarcinoma (Fig. 2a). The tumors consisted of rather uniform but differently sized acinar structures separated by little intervening stroma. The acini were formed of cuboidal malignant epithelial cells, mostly arranged in one layer, only rarely in more layers. The cells and their nuclei showed only moderate

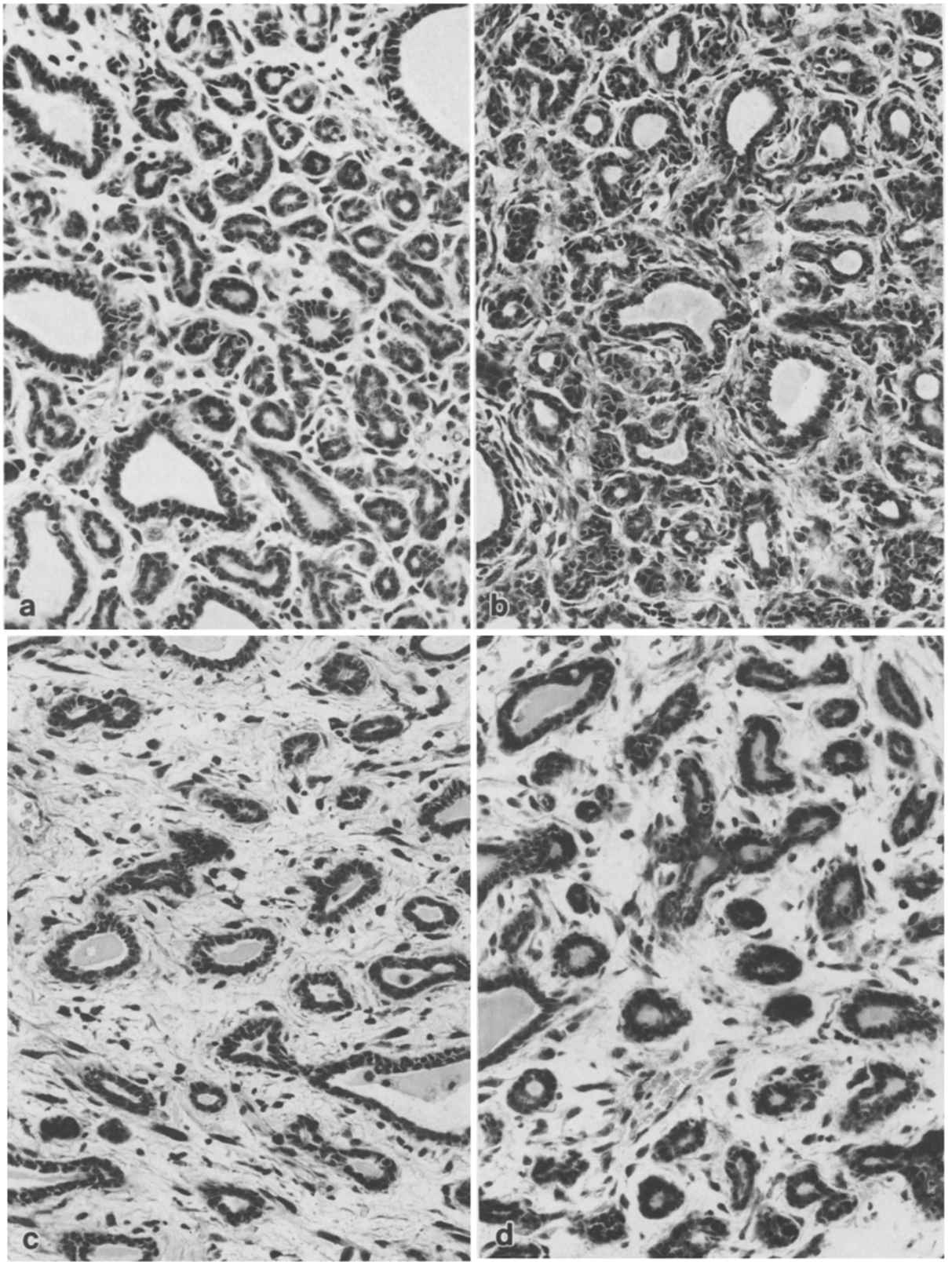

Fig. 2a–d. R3327 Dunning prostatic carcinoma. **a** Control, **b** castration + adrenal androgens, **c** castration, **d** castration + adrenal androgens without DHEA-S. H&E, ×240

variations in shape, size, and staining. There was little mitotic activity. The stroma consisted of connective tissue with collagenic fibers and capillaries. The central parts of the tumors showed areas with necrosis and liquefaction, but there were no hemorrhages.

The morphology of tumors of castrated rats treated by application of adrenal androgens was similar to that of tumors of the intact control group, and this reflects the situation of the tumor size (Fig. 2b).

Tumors of castrated rats without any further therapy showed an evident change of their morphology (Fig. 2c). Castration caused an absolute and relative decrease in the quantity of the epithelial portion of the tumors. There was a decrease in the number and in the mean dimension of the acini, which also showed a loss of their uniformity. On the other hand there was a relative increase in the quantity of the stromal portion, which also contained an excessive amount of tissue fluid. Many areas of the tumors showed degeneration and necrosis of cells and nuclei.

Application of adrenal androgens without DHEA-S in castrated rats led to a tumor morphology which was more similar to that of untreated castrated rats (Fig. 2d). The tumors consisted of differently sized acini, which were separated by thick layers of edematous connective tissue.

To quantify these morphological changes, morphometry of the tumors was performed. Volume density data of control showed a 59% portion of epithelial and a 41% fraction of stromal elements. After castration, this ratio was reversed. Application of adrenal androgens almost reversed this ratio again, whereas treatment with adrenal androgens without DHEA-S gave results more similar to those from castrated rats (Table 6).

Discussion

The role of adrenal androgens in tumor progression after surgical or chemical castration has been a point of controversial discussion. Labrie and his group used total androgen (blockade of androgens of testicular and adrenal origin) to prevent a relapse. According to their publications, all cells of the prostatic carcinoma are hormone sensitive but to different degrees [15]. The total androgen blockade proposed by the group of Labrie led in first experiments to a 100% objective response [15]. Labrie used the combination of a luteinizing hormone releasing hormone (LHRH) analogue and a pure antiandrogen (Anandron and flutamide).

The results obtained in the experiment with R3327 clearly demonstrate that the application of a mixture of adrenal androgens induces tumor progression in castrated, tumor-bearing rats to the intact control level. The stimulatory effect on accessory sex organ weights was much lower than that on the tumor. By implanting testosterone capsules in normal and R3327 G or H bearing rats it was demonstrated that the G and H tumors are both more sensitive to androgenic stimulation than is the normal prostate [6].

The importance of adrenal androgens in prostatic cancer is mainly related to their intraprostatic conversion to T and DHT [16]. In humans, skin [24], blood [3], and other tissues are able to metabolize adrenal androgens to T and DHT. The application of the adrenal androgen mixture in our experiments drastically elevated plasma levels of T, a clear hint for a similar conversion of the applied adrenal androgens in tissues other than the prostate. The high DHT level in the tumor tissue of adrenal androgen-treated rats is therefore due to the high circulating levels of T. The DHEA-S concentration was the highest of all adrenal androgens used in the mixture, but there was no difference in effect on plasma level of T and tissue levels of T and DHT in comparison with the group treated with the adrenal androgen mixture without DHEA-S. The conversion of DHEA-S to T or DHT is therefore only of minor importance for tumor progression. Stimulation of normal prostates could only be achieved by using the mixture of adrenal androgens; DHEA-S alone had no effect.

Our results indicate that tumor progression is induced after castration in the case of a high circulating T level. The question arises whether the T level in humans after castration is high enough to cause tumor progression. Only then would the use of a total androgen blockade be a more effective therapy. A compensatory increase in the T level after removing the testes, however, was not observed [10]. Experiments using normal prostates [13, 14] as well as R3327 H tumors [6, 23] demonstrated that there is a certain threshold level of T or DHT necessary for growth. Once the plasma T levels are below this critical threshold, further hormonal manipulation would be rather futile.

The Dunning R3327 system of rat prostatic adenocarcinomas has been demonstrated to mimic many of the important properties of human prostatic cancer [12]. There is much evidence that a similar situation exists in humans as seen in the experiments using the R3327 model system.

Newer trials using complete androgen withdrawal in comparison with partial withdrawal could not match the results achieved by Labrie and his group [15].

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