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After radiotherapy testosterone stimulation is unable to increase growth in the Dunning R3327-PAP prostate tumour

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Abstract A study was carried out to investigate whether testosterone treatment is able to influence tumour growth in a rat prostatic adenocarcinoma previously treated with castration and high-dose fractionated irradiation. Copenhagen × Fisher rats bearing the androgen-sensitive, well-differentiated Dunning R3327-PAP tumour were castrated and thereafter treated with external beam radiation with photons from a 4-MV linear accelerator. One month after irradiation, substitution with subcutaneous testosterone was started. Tumour volumes and rat weights were monitored up to 256 days after castration, and at the end of the study a microscopic analysis of the tumours was performed. Irradiation delayed tumour growth as compared with untreated tumours. Castration delayed tumour growth, but a hormone-refractory relapse to doubled tumour volume was seen within 45 days. If testosterone was added after castration, the tumours grew rapidly. However, testosterone failed to increase tumour growth when given to rats treated with orchiectomy and irradiation. Histological examination showed that the irradiated tumours still contained tumour epithelial cells, but these cells apparently do not respond to testosterone stimulation. The well-differentiated and androgen-sensitive rat prostatic adenocarcinoma did not grow after irradiation despite stimulation with testosterone. This indicates that the malignant cells lose their androgen sensitivity after high-dose irradiation.

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Introduction

Combining androgen deprivation and radiotherapy has been shown to give therapeutic advantages both experimentally [5, 8] and in clinical trials [1, 6, 11, 13] in the management of localised and locally advanced prostate cancer. The optimal duration and sequence of androgen suppression remain to be defined, however.

In the clinical management of prostate cancer patients treated with external beam radiotherapy, there is also controversy about the clinical importance of residual cancer cells in post-treatment biopsies [2, 9, 14]. Nowadays most authors strive for negative biopsies 12–24 months after irradiation, and in this respect theresults have improved as a result of innovations such as dose escalation with three-dimensional conformal radiation therapy [17], combination with androgen deprivation [11] and interstitial radiation [16]. However, the malignant potential of remaining cells is unknown. It is also unknown whether these cells are androgen sensitive, and therefore there is uncertainty about the clinical significance of normal serum testosterone levels. It is of interest to know whether and when serum testosterone levels should be allowed to return to normal, when radiotherapy is combined with various types of androgen deprivation therapies. This is also a topic of interest regarding the trends in hormone therapy to avoid adverse effects of castration by using nonsteroidal antiandrogens as monotherapy [12]. We therefore investigated whether testosterone is able to stimulate growth in previously irradiated Dunning R3327-PAP rat prostatic tumours.

Materials and methods

Animals

Tumour tissue from Dunning R3327-PAP rat prostatic adenocarcinoma was implanted as an approximately 1-mm^3 core subcutaneously into the right flank of Copenhagen × Fisher F1 hybrid male rats 10 weeks after birth. This well-differentiated, androgen-sensitive tumour subline was originally obtained from Dr. N.H. Altman (Organ System Program of the National Cancer Institute, USA). Rats were obtained from Møllegaard, Denmark and housed in a controlled environment (12 h light/12 h dark) where they were supplied with pelleted food and water ad libitum. They were weighed using standard laboratory procedure each time tumour volumes were measured (see below). The animal ethics committee at Umeå University approved the study.

Treatment groups

The animals in this experiment had a single tumour in their right flank. The tumours were stratified for tumour volume, randomly allocated into five groups and treated as shown in Table 1.

Orchiectomy

The animals were surgically castrated 4 months after tumour inoculation, when the tumours had a volume of about 1 cm^3. Bilateral orchiectomy was performed through a mid-line scrotal incision. The rats were anaesthetised with intraperitoneal injections (3 ml/kg body weight) of a cocktail consisting of 0.079 mg fentanyl, 2.5 mg fluanisone and 1.25 mg midazolam in 1 ml of sterile water. After this procedure the animals recovered well within a few hours.

Radiotherapy

External beam radiation was performed 1 month after surgical castration. The radiation dose of 50 Gy was fractionated into 10 Gy on five consecutive days and given with a photon beam from a 4-MV linear accelerator. The dose rate was approximately 2.5 Gy/min and the source-to-skin distance 70 cm. The radiation field size was chosen to give at least a 5 mm margin around the palpable tumour. During the radiation procedure the non-anaesthetised animals were kept in a metallic frame by strong cotton net. The animals were continuously observed through a video camera and, if they moved, the irradiation was stopped and the position of the tumour in the radiation field was re-adjusted if necessary. Two animals were oriented in opposite directions and treated in the same field. In order to achieve an adequate radiation dose in the tumour a Plexiglas shield was used to eliminate the build-up and water-equivalent silicone material was used to give backscatter. The dosimetry referred to basic measurements and additional controls were done with calibrated diodes in a rat phantom of wax in the same geometry as during the experimental irradiation. A high radiation dose [7] was chosen for maximal tumour effect. The rats used as controls were not irradiated but were trained in the same frame for equally long periods and thus sham-treated.

Table 1 Treatment groups

Treatment group	Abbreviation for treatment group	No. of tumours
Castration + radiation + testosterone	A + RT + T	14
Castration + radiation	A + RT	10
Castration + testosterone	A + T	10
Castration only	A	10
Controls (no treatment)	Control	8

Testosterone substitution

The testosterone substitutions were started 1 month after the completion of radiotherapy. A subcutaneous dose of 12.5 mg of testosterone enanthat (Testoviron-Depot, Schering) was given twice weekly during the first 4 weeks and then once weekly, until the tumours were taken for morphological analysis.

Tumour volumes

The same investigator measured tumour diameters with callipers on non-anaesthetised animals. Three perpendicular diameters ($D1$, $D2$, $D3$) were measured before treatment and at 2–3 week intervals after treatment. Tumour volumes were estimated by the formula for an ellipsoidal mass: $\pi/6 \times D1 \times D2 \times D3$. The tumour volume at castration was defined as 100%. From that value, the relative growth of the tumours was followed up regularly.

Morphological analysis

At the end of the experiment the rats were killed by decapitation. Three randomly chosen samples from each tumour were fixed in 4% buffered formaldehyde solution and embedded in paraffin. Four-micrometre (4-μm) thick sections were stained with haematoxylin and eosin. A morphometric analysis was performed using light microscopy at ×400 magnification, and the volume densities of tumour epithelium, stroma and lumina were calculated as previously described [10].

Results

The animals

Orchiectomy was well tolerated by the animals. There was no death from anaesthesia and no notable weight loss.

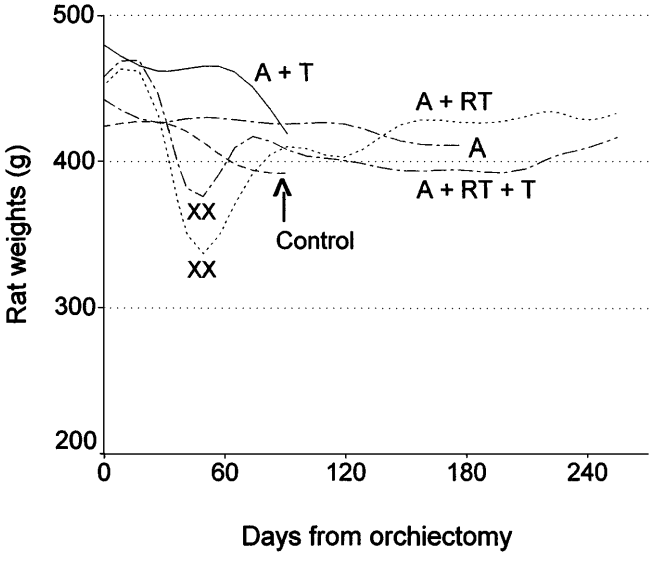

Fig. 1 Mean animal weights of different treatment categories. Significant weight losses in both the irradiated groups (A + RT + T and A + RT) compared with the respective weights before irradiation are indicated by *double crosses* ($P < 0.01$, Wilcoxon signed-rank test). *A* androgen ablation (orchiectomy), *RT* external beam radiation, *T* testosterone substitution, *Control* no treatment

The changes in the animals' weights are presented in Fig. 1. Before the start of treatment there was no significant difference in body weight between the treatment groups ($P = 0.12$, Kruskal-Wallis one-way ANOVA). The high-dose fractionated radiotherapy 4 weeks after the initial orchiectomy resulted in a significant body weight loss in both groups. They started to regain weight after 2 weeks. Some animals in both irradiated groups suffered from cachexy at various times after irradiation, and had to be killed before completion of the experiment. Thus, their tumours were excluded from further growth follow-up and morphological evaluation.

At the end of the experiment the prostate glands of the irradiated and testosterone-stimulated rats (group A + RT + T) were hyperplastic, averaging 2.3 g in weight, while atrophic prostate remnants found in the other irradiated rats (A + RT) had a mean weight of 0.2 g. The normal prostate weight in intact rats is about 0.5 g [4].

Tumour growth

The tumour volumes of the various treatment groups before orchiectomy and on days 92, 178 and 256 are presented in Fig. 2. Before orchiectomy the size of the tumours in the various treatment groups was not significantly different ($P = 0.31$, Kruskal-Wallis one-way ANOVA). There was no significant tumour shrinkage in comparison with the initial volume in any of the treatment groups, as tested with the Wilcoxon signed-rank test. It has earlier been shown that the volume of the Dunning tumour does not decrease after castration only [10]. In this study the castration-only group relapsed to 200% of the initial volume within 45 days.

Comparison between groups

Irradiation clearly delayed tumour growth as compared with untreated tumours, tumours treated with castration only and those substituted with testosterone 2 months after orchiectomy (Fig. 3). In castrated animals testosterone substitution led to fast tumour growth parallel to the tumour growth in untreated animals.

Testosterone substitution did not stimulate growth of tumours exposed to high-dose irradiation, because there was no difference in tumour growth as compared with non-substituted, irradiated tumours (Fig. 3). The follow-up was extended to 200 days after initiation of testosterone substitution, when both irradiated tumour groups had started to regrow.

Morphology

In the morphological analysis of tumours from the endpoint of the study, there was no statistically significant difference between different cell compartments in the two irradiated groups, in accordance with the similarity in tumour growth curves. Tumours excised at the end of the study (groups A + RT and A + RT + T) consisted of about 40% epithelial cells, 55% tumour stroma, and 5% lumina. Thus, a substantial amount of tumour epithelial cells were seen about 7 months after irradiation.

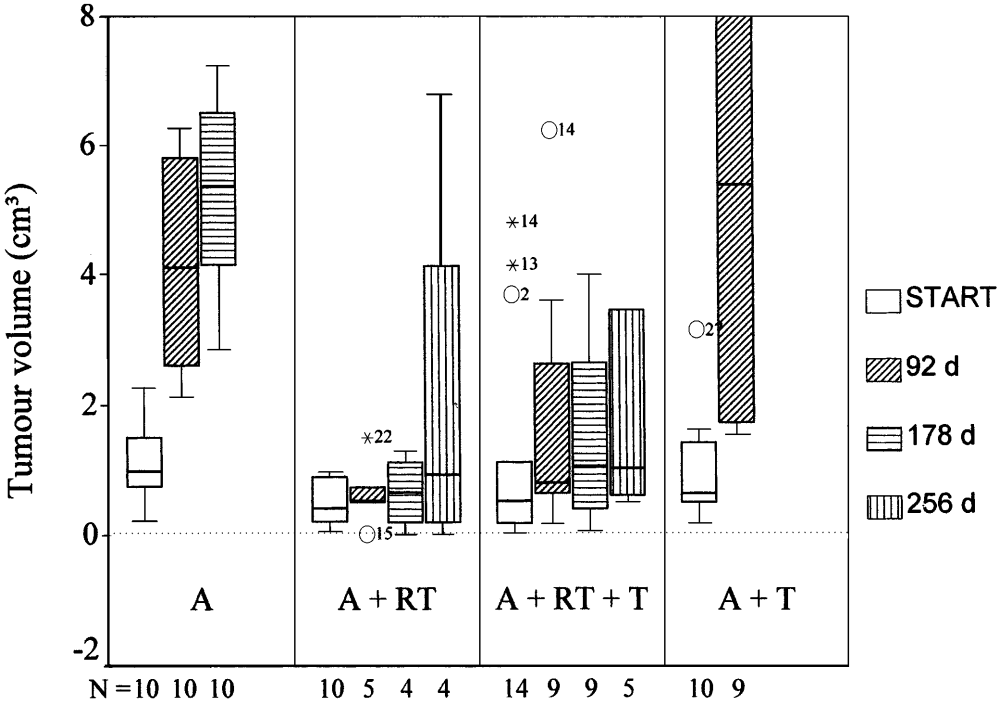

Fig. 2 Boxplot showing the tumour volumes in cubic centimeters in the various treatment groups at the start of the experiment and at 92, 178 and 256 days after orchiectomy. The number of tumours included after respective follow-up intervals is indicated below the frame. Each *box* represents the interquartile range (from 25% to 75% of the values), with the median value marked inside, and the maximum and minimum values marked outside. *Circles* represent outliers, i.e. cases with values between 1.5 and 3 box-lengths from the upper or lower edge of the box; *asterisks* are extreme outliers, i.e. cases with values more than 3 box-lengths from the upper or lower edge of the box. *A* androgen ablation (orchiectomy), *RT* external beam radiation, *T* testosterone substitution

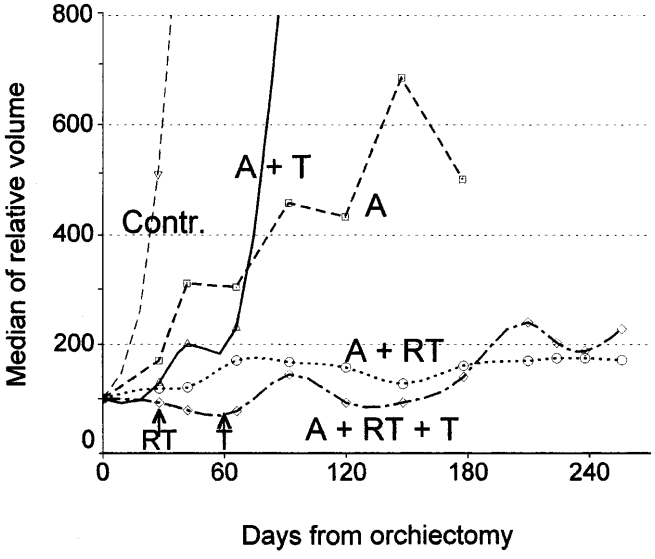

Fig. 3 The relative change in tumour volume starting from the day of castration. The start of radiotherapy (*RT*) and testosterone substitution (*T*) is indicated with *arrows*. *A* androgen ablation (orchiectomy), *RT* external beam radiation, *T* testosterone substitution, *Control* no treatment

Discussion

In this study we have shown that testosterone treatment fails to stimulate tumour growth in androgen-sensitive prostatic adenocarcinoma of previously castrated rats exposed to fractionated irradiation. In castrated rats about 0.1 mg/day of testosterone is needed to normalise serum testosterone [3]. The testosterone dose used in this experiment was considerably higher and it induced a 4-fold increase in the ventral prostate gland, but in spite of that the irradiated tumours did not grow faster than those not stimulated by testosterone. In contrast, this dose of testosterone resulted in a major increase in tumour growth in castrated animals. It therefore appears that the tumour cells remaining after irradiation have lost their androgen sensitivity. If the present experimental finding is extrapolated to the clinical situation, it raises the question whether, after radiation treatment, it really is beneficial to decrease testosterone below physiological levels in patients given radiotherapy as first-line treatment for localised prostate cancer.

After castration the tumours relapsed early in a hormone-refractory way in this experiment. That is in stark contrast to our previous finding of considerably delayed regrowth of the Dunning R3327-PAP tumour in castrated rats [5]. We interpret this as a variation in androgen sensitivity of experimental tumour cells from different tumour batches, thus emphasising the importance of using adequate controls from the same tumour batch in this type of experimental work.

The clinical importance of normal levels of circulating serum testosterone after radiotherapy of prostate cancer is not well studied. There are no clinical data on testosterone stimulation after curatively intended radiotherapy. In a study by Zagars et al. [15] high serum testosterone levels were not associated with acceleration of postradiation serum postate-specific antigen (PSA) kinetics, suggesting that human prostate cancer cells also may lose their androgen sensitivity after high-dose irradiation. In another recently published analysis of serum PSA levels at 24 months after radiotherapy there was no additive effect of intra- and post-treatment androgen deprivation as compared with 3 months of neoadjuvant total androgen blockade without further androgen deprivation [11], again suggesting a low androgen sensitivity in irradiated prostate cancer cells. In clinical practice there is a tendency to rely more on serum PSA levels than postradiation biopsy findings because of the low probability of finding small areas of tumour cells with a limited number of biopsies and the unclear malignant potential of remaining tumour cells. The present findings suggest that it could be of value to test whether the remaining tumour cells in post-irradiation biopsies respond to testosterone in vitro or not, and to use second-line androgen ablation therapy only in those tumours with proven testosterone sensitivity. It should, however, be noted that some patients given radiotherapy for presumed localised disease have unrecognisable distant metastases. Although tumour cells in the prostate may lose their ability to grow in response to testosterone after irradiation, the tumour cells in the micrometastases may still be testosterone sensitive and, thus, responsive to androgen ablation therapy.

Conclusion

The cells remaining in experimental prostate tumours after combined androgen ablation therapy and irradiation may have lost their ability to grow and divide in response to testosterone stimulation. It remains to be clarified whether this finding is relevant to clinical practice.

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