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Decrease in the AgNOR Number in Dunning R3327 Prostate Cancers After Treatment with an Agonist and Antagonist of Luteinizing Hormone-releasing Hormone

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The argyrophilic staining of the nucleolar organizer region (AgNOR) in cells of Dunning R3327 rat prostate tumors was studied and the effect of hormonal treatments on their appearance was analyzed. The nuclei of the control tumor cells contained 4.1 ± 0.17 AgNOR granules. Treatment of rats for 8 weeks with luteinizing hormone-releasing hormone (LH-RH) agonist (D-Trp-6-LH-RH) and antagonist SB-75 induced a marked inhibition of tumor growth and decreased significantly ($P < 0.01$) the number of AgNORs in the tumors to 2.89 ± 0.10 AgNOR granules/cell in the group given the agonist and to 2.82 ± 0.10 after therapy with the highest dose of the antagonist. A reduced AgNOR number (3.14 ± 0.16) also was found after 3 days of treatment with SB-75 ($P < 0.05$), but the AgNORs returned to near control values 1 week after the short-term therapy, showing the reversibility of these changes. These results suggest that the AgNOR method, which was widely tested on human tumors in the past few years, can be a valuable technique in experimental tumor pathology and useful in the evaluation of the effects of various treatments. (Am J Pathol 1991, 138:1273–1277)

In the last few years, an increasing interest was focused on the proteins associated with nucleolus organizer regions (NOR) and the AgNOR method. Nucleolus organizer regions are the sites of ribosomal genes, associated with acidic nonhistone proteins that can bind silver ions, thus allowing NOR to be visualized by the argyrophilic staining method.[1] The exact nature and significance of these proteins are uncertain, but an association with nucleolar phosphoproteins and RNA polymerase I has been suggested.[2,3] They could play an important role in the rDNA transcription,[4] but no correlation between the quantity of NORs and ribosomal gene activity was found in other studies.[5] The number, distribution, and size of AgNORs may reflect changes in cellular activity[5] and could be related to cell turnover.[6] A difference in AgNOR count has been found between benign and malignant tumors. Tumors of high-grade malignancy have smaller, more numerous AgNORs than those that are less malignant.[7] The AgNOR method has been found to be an efficient tool for differentiating malignant and benign pathologic processes of certain organs, but no significant differences could be found between benign and malignant tumors at other sites. Crocker and colleagues[7] have done much work on the differential diagnostic evaluation of the AgNOR method. Recently they made an exhaustive general survey about the topic.[7] Thamm and Page[8] and Quinn and Wright[9] critically reviewed the significance of the AgNOR method in tumor histopathology.

In addition to the field of oncology, an increase of AgNOR numbers has been demonstrated in cells after hormonal stimulation[10] and in rat pituitary corticotrophs after adrenalectomy.[11] In light of changes in AgNORs in various tumors and in tissues after hormonal stimulation, we thought that it was of interest to investigate the appearance of AgNOR in tumors after hormonal therapy. In this study, we examined the effects of luteinizing hormone-releasing hormone (LH-RH) agonist D-Trp-6-LH-RH and antagonist SB-75 on the AgNOR count in Dunning R3327 hormone-dependent rat prostate cancer.

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Materials and Methods

Peptides

The LH-RH analog D-Trp-6-LH-RH (pyroGlu-His-Trp-Ser-Tyr-D-Trp-Leu-Arg-Pro-Gly-NH_2) was synthesized by solid-phase methods and supplied by Debiopharm (Lausanne, Switzerland). Microcapsule formulation of this agonist in biodegradable poly(DL-lactide-co-glycolide) was prepared by Dr. P. Orsolini at Cytotech (Martigny, Switzerland) using a phase-separation process. This delayed release formulation, in an aliquot of 36 mg, maintained a continuous liberation of approximately 25 μg/day of the analog for 30 days. The LH-RH antagonist [Ac-D-Nal(2)', D-Phe(4Cl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10})]LH-RH (SB-75), originally synthesized in our laboratory,[12] was made by Asta Pharma Co. (Frankfurt, FRG) using solid-phase methods. Microcapsules of SB-75 in poly(DL-lactide-co-glycolide) for sustained release, also prepared by Dr. P. Orsolini, were designed to liberate about 23.8, 47.6, and 71.4 μg/day from aliquots of 25, 50, and 75 mg, respectively. This batch of SB-75 microcapsules was injected every 3 weeks. Both types of microcapsules were suspended in 0.7 ml injection vehicle (2% carboxymethyl [CM] cellulose and 1% Tween 80 in water) for intramuscular injection.

Animals and Tumors

Male (Copenhagen × Fisher) F_1 rats, bearing the R3327 H Dunning rat prostate adenocarcinoma, were a gift from Dr. Norman Altman (Papanicolaou Cancer Research Institute, Miami, FL). The rats were housed four per cage at the Animal Research Facility of our institution in an air-conditioned room at 21° ± 1°C and 55% ± 5% humidity. The animals were kept under an automatic light–darkness schedule (12 to 12 hours) and given Rodent Laboratory Chow 50001 (Purina Mills, Inc., St. Louis, MO) and tap water *ad libitum*. Tumors were measured weekly by a caliper and volume was calculated as described previously.[13]

Experimental Protocol

Nineteen weeks after implantation, the tumors were well developed and the animals received treatments according to the following schedules.

A. Chronic (8 weeks) treatment, with the following groups: 1) injection vehicle only; 2) D-Trp-6-LH-RH microcapsules 36 mg/animal administered on days 0 and 28; 3) SB-75 microcapsules 25 mg/animal on days 0, 21, and 42; 4) SB-75 microcapsules 50 mg/animal on days 0, 21, and 42; 5) SB-75 microcapsules 75 mg/animal on days 0, 21, and 42. Each group contained seven rats and the injections were given intramuscularly.
B. Short-term (3 days) treatment, with the following groups: 6 and 8) injection vehicle only; 7 and 9) SB-75 200 μg/animal once a day, administered subcutaneously.

Animals in groups 1 to 5 were killed 8 weeks after the start of the treatments, animals in groups 6 and 7 were killed 2 hours after the last injection, and the rats in groups 8 and 9 were killed 7 days after the last treatment.

Pathologic Procedures

Under Metofane (Pitmann Moors, Washington Crossing, NJ) anesthesia, the rats were decapitated. Tumor tissue was fixed in 10% buffered formalin. Specimens were embedded in Paraplast (Oxford Labware, St. Louis, MO) and 4-μm–thick sections were cut. For the determination of the AgNOR numbers, Chiu's staining method[14] was used. However, at the end of the process, before dehydration, the sections were placed in 5% sodium thiosulfate solution for 10 minutes and washed with distilled water again. The granules in 50 nuclei of the glandular epithelial cells of each tumor were counted and the mean AgNOR number per nucleus was calculated. The computer-assisted Duncan's multiple range test was used for the statistical analysis of the data.

Results

Tumor weights and tumor volumes as well as the weight of testicles measured at the end of the chronic experiment are shown in Table 1. D-Trp-6-LH-RH and SB-75 in doses of 47.6 μg/day and 71.4 μg/day exerted a marked inhibitory effect on tumor growth about to the same degree. The same groups also showed decreased weights of testicles and histologically, a total inhibition of spermatogenesis. The lowest dose of SB-75 (23.8 μg/day) had a smaller, but still significant, inhibitory effect on tumor growth and caused partial inhibition of spermatogenesis. Other oncologic and endocrine details of this study and pathologic results are fully described elsewhere.[15,16]

Histologically all the tumors were well-differentiated adenocarcinomas. The basic histologic pattern was similar in all groups, but regressive changes, including an increased incidence of apoptosis, reduced number of mitoses and increased amount of connective tissue stroma could be observed in the tumors of animals treated for 8 weeks.

Table 1. *The Effect of Treatment with LH-RH Analogs on Tumor Weight and Volume and Weights of Testicles in Rats with Dunning Prostate R-3327 Cancer*

Group	Tumor volume (mm^3)	Tumor weight (g)	Weight of testes (g)
1. Control	6815.5 ± 908.1	6.075 ± 0.89	2.48 ± 0.27
2. D-Trp-6-LH-RH: 25 μg/day	1687.5 ± 338.9†	1.270 ± 0.23†	0.53 ± 0.02†
3. SB-75: 24 μg/day	4692.9 ± 531.9*	4.019 ± 0.50*	1.85 ± 0.26*
4. SB-75: 48 μg/day	2551.1 ± 560.7†	2.304 ± 0.57†	0.52 ± 0.03†
5. SB-75:71 μg/day	1515.2 ± 383.3†	1.505 ± 0.45†	0.53 ± 0.02†

Values are means ± S.E.
* $P < 0.05$.
† $P < 0.01$.

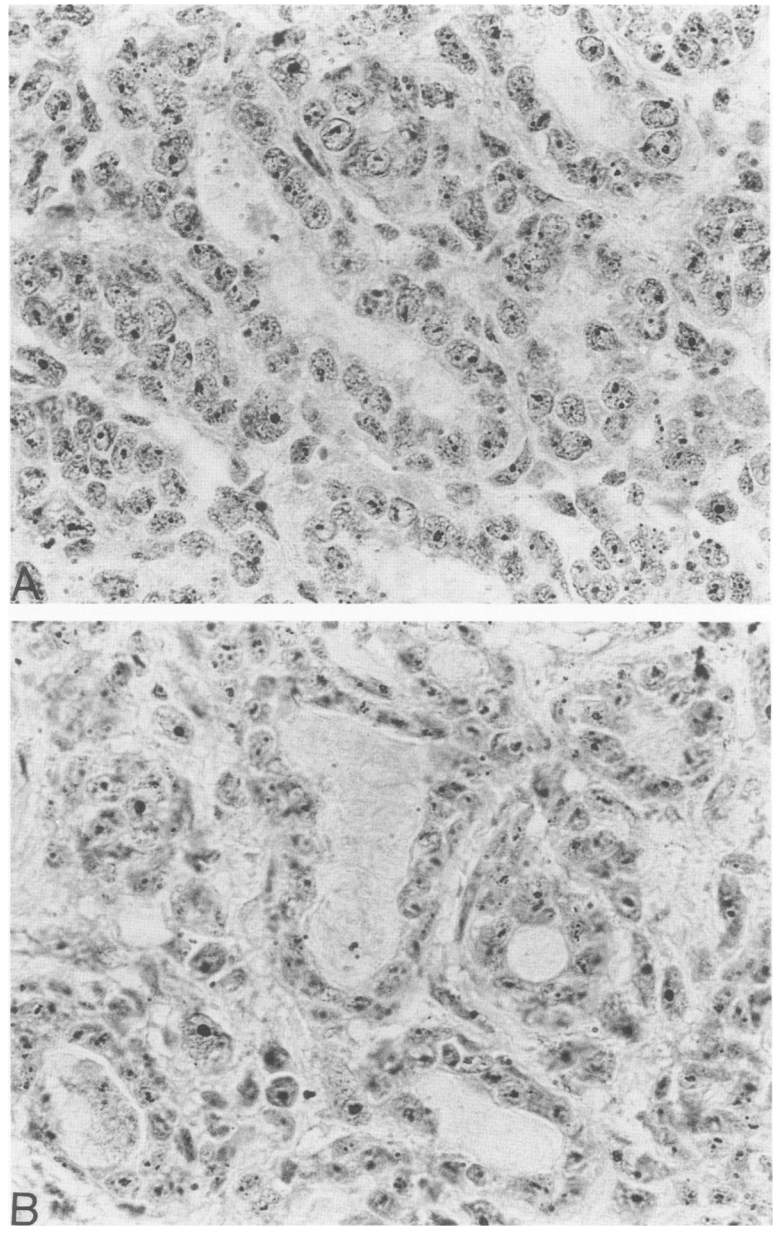

Figure 1. *AgNORs in control Dunning prostate cancer* (**A**) *and after treatment with 48 μg/day SB-75 for 8 weeks* (**B**), ×800.

Table 2. *AgNOR Counts in Dunning Prostate Cancers After Treatment with LH-RH Analogs*

Groups	Mean	SE	%
A. Chronic treatment (8 weeks) using microcapsule formulations			
1. Control	4.098	0.168	100.0
2. D-Trp-6-LH-RH: 25 μg/day	2.886†	0.097	70.4
3. SB-75: 24 μg/day	3.043†	0.083	74.2
4. SB-75: 48 μg/day	2.863†	0.064	69.9
5. SB-75: 71 g/day	2.823†	0.095	68.9
B. Short-term (3 days) treatment using daily injection			
6. Control	3.745	0.097	100.0
7. SB-75: 200 μg/day	3.136*	0.163	83.7
8. Control: 1 week later	3.820	0.121	102.0
9. SB-75: 1 week later	3.575	0.163	95.5

SE, standard error.
* $P < 0.05$.
† $P < 0.01$.

The AgNOR granules were clearly visible in the nuclei of tumor cells. They were smaller black granules dispersed in the nucleus, sometimes in or attached to the nucleolus. Larger black spots could be seen only in a few nuclei. The size and shape of the granules did not differ in the various groups (Figure 1). A significant difference in the number of AgNORs could be demonstrated between the treated and untreated tumors (Table 2, Figure 2). The AgNOR count decreased in all groups treated with the LH-RH analogs for 8 weeks, but its reduction was somewhat less for the smallest dose of SB-75. A reduced number of AgNORs also was observable after treatment with SB-75 for 3 days (group 7) and the number returned to near control values 1 week after the short-term treatment (group 9) (Table 2).

Discussion

Dunning prostate carcinoma is a good model of human prostate adenocarcinoma; its hormonal dependency makes it very useful for testing endocrine manipulations, including the use of LH-RH analogs.[13,17,18] In many previous studies, a higher AgNOR count was found in malignant tumor cells than in benign alterations or normal tissues. Although the AgNOR method can be helpful in the differential diagnosis of some human tumors, its wide use in routine diagnosis is limited by the overlap when individual cases are considered. The significance of NORs is not well understood. Their count depends on the stage of cell cycle.[9] In tumors, malignant transformation, the degree of differentiation, and the proliferation rate of cells may be reflected in the AgNORs.[4,6,19] In nontumorous tissues, the ploidy,[20] hyperplasia, and increased synthetic and metabolic activity of the cells may influence the AgNORs.[11] A decrease of metaphasic AgNOR activity was observed in a tumor cell line with chromosome aberrations after treatment with a differentiating agent.[21] Chromosomal changes, related to growth rate and differentiation, also were found in Dunning prostate cancer.[22]

The growth inhibition of the Dunning R3327 hormone-dependent prostate cancer induced by treatment with D-Trp-6-LH-RH and SB-75 was observed in our study.[15] The slower growth rate was accompanied by histologic signs of regression and decreased malignancy of the tumors, lower number of mitoses, increased apoptosis, and reduced parenchyma/stroma ratio. The reduced number of AgNORs in the treated tumors in our study, together with the slower growth rate and the overall histologic condition, suggest that the Dunning R3327 prostate cancer becomes a more differentiated tumor after treatment with the agonist D-Trp-6-LH-RH and antagonist SB-75. The acute (short-term) treatment with SB-75 resulted in a smaller decrease of AgNOR numbers and the values returned close to control counts 1 week after the treatment. Thus the reversibility of hormonal suppression on tumor cells could be demonstrated.

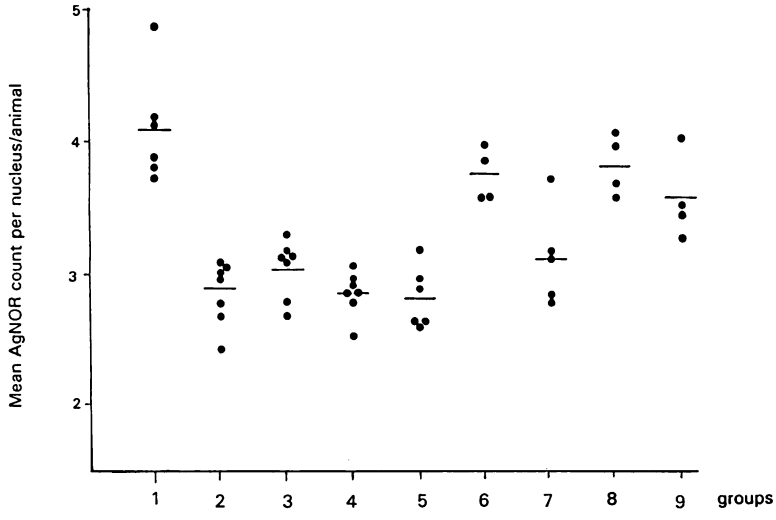

Figure 2. *Scattergram of AgNOR counts in Dunning prostate tumors. Groups: 1, Control; 2, D-Trp-6-LH-RH; 3, SB-75 24 μg/day; 4, SB-75 48 μg/day; 5, SB-75 71 μg/day; 6, Control; 7, SB-75; 200 μg/day for 3 days; 8, Control 1 week later; and 9, 1 week after the short-term treatment with SB-75.*

Experimental tumors are more homogenous than individual human tumors with respect to their AgNOR content. Experimentally this method need not be used for the evaluation of single cases, as for instance in differential diagnosis in surgical pathology. Thus we conclude that the study of the AgNOR count in experimental cancers and the evaluation of their changes after various antitumor treatments can be valuable methods for the determination of the malignant potential of the tumors as well as of the efficacy of the therapy.

Similarly the AgNOR method may be a helpful tool also in human pathology for determination of the efficacy of nonsurgical treatments in some types of tumors, even in aspirated biopsy material. In such cases, the tumor tissue obtained from the same patient allows for a good comparison without great variations of individual data.

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