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Cellular proliferation and/or differentiation can condition modifications in the expression of AgNORs in rat anterior pituitary cells during growth

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Summary. The expression of argyrophilic nucleolar organizer regions (AgNORs) was studied in the anterior lobe of the hypophysis of 1-, 7-, 14-, 28- and 80-day-old rats. Mean area and numbers of AgNORs per nucleus decreased during this period with a simultaneous increase of both nuclear area and mean AgNOR-particle area. Comparison of our data on AgNOR expression with previously reported data on the proliferative activity and cell maturation and differentiation of anterior pituitary cells during a similar period led us to conclude that, at least in our experimental model, variations in the expression of AgNORs are more related to phenomena of cellular maturation than to proliferative activity.

Key words: AgNORs, Anterior pituitary, Maturation, Rat

Introduction

Nucleolar organizer regions (NORs) contain DNA loops that code for ribosomal RNA (Schwarzacher and Wachtler, 1983). NORs can be identified in the nuclei of interphase cells by a silver staining technique than selectively stains some acidic proteins (AgNORs) associated to ribosomal genes (Ploton et al., 1986). Since the assessment of AgNORs was first introduced into morphological oncology (Crocker and Nar, 1987) many studies have been carried out in an attempt to correlate changes in the expression of AgNORs with the diagnosis of malignancy (Crocker, 1990; Underwood, 1992).

The exact significance of changes in the expression of AgNORs is not yet clear. The number of AgNORs has been reported to be a marker for cellular differentiation (Smetana and Likovsky, 1984). Furthermore, the quantity of AgNORs may be related to metabolic functions (De Capoa et al., 1985; Peebles and McNicol, 1989). Indeed, many studies have related AgNOR expression to cellular proliferation (Crocker et al., 1988; Hall et al., 1988; Derenzini et al., 1989). Interestingly, most of these studies in which AgNOR expression is related to cellular proliferation have been performed on tumor samples, where the influence of the degree of differentiation or dedifferentiation of tumor cells on the expression of AgNORS is difficult to evaluate.

The aim of the present study was to attempt to clarify the meaning of the expression of NORs in non-pathological cells by analyzing AgNORs during postnatal development of the pars distalis of the hypophysis, a glandular tissue well documented as regards its differentiation and proliferation (Dearden and Holmes, 1976; Carbajo-Pérez and Watanabe, 1990; Carbajo et al., 1992).

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Materials and methods

Sprague-Dawley rats were used. Animals were caged by litters until the day of experiment. All groups in the experiment included 4 females. Groups of 1-, 7-, 14-, 28- and 80-day-old rats were killed by cervical dislocation. The cranial vault was cut away and the brain removed. The pituitary gland was then carefully isolated with the aid of fine forceps and a dissecting microscope. All specimens were formalin-fixed and paraffin-embedded. Pituitaries were sectioned at a thickness of 3 μm along the frontal plane.

After deparaffinization, AgNORs were revealed as described by Ploton et al. (1982) with minor modifications. Glass slides were throughly washed in distilled water and incubated for 20 min at 40 °C in the dark with the silver colloid staining solution. The staining solution was prepared by dissolving gelatin at 20 g/l in 10 g/l aqueous formic acid and mixing with twice the volume of 500 g/l aqueous silver nitrate solution. Samples were washed and mounted with DPX.

AgNORS were studied with an MIP image analyzer. Four slides per group, 25 cells per slide from 8

microscope fields selected at random were studied with a x 100 aberration-free objective lens (Carl Zeiss Jena, Germany). The image projected onto the CCD used for image capture was mapped into a 512 x 512 x 24 bit memory. The content of the frame memory is equivalent to 3235 μm^2 of the histological preparation. The nuclear area, the total area and numbers of AgNORs per cell as well as the mean AgNOR-particle area was calculated.

Comparison of individual variables was performed by ANOVA to a level of significance of 5%. The Fisher test was used for two group comparisons for the relationships among the different AgNOR-derived parameters.

Results

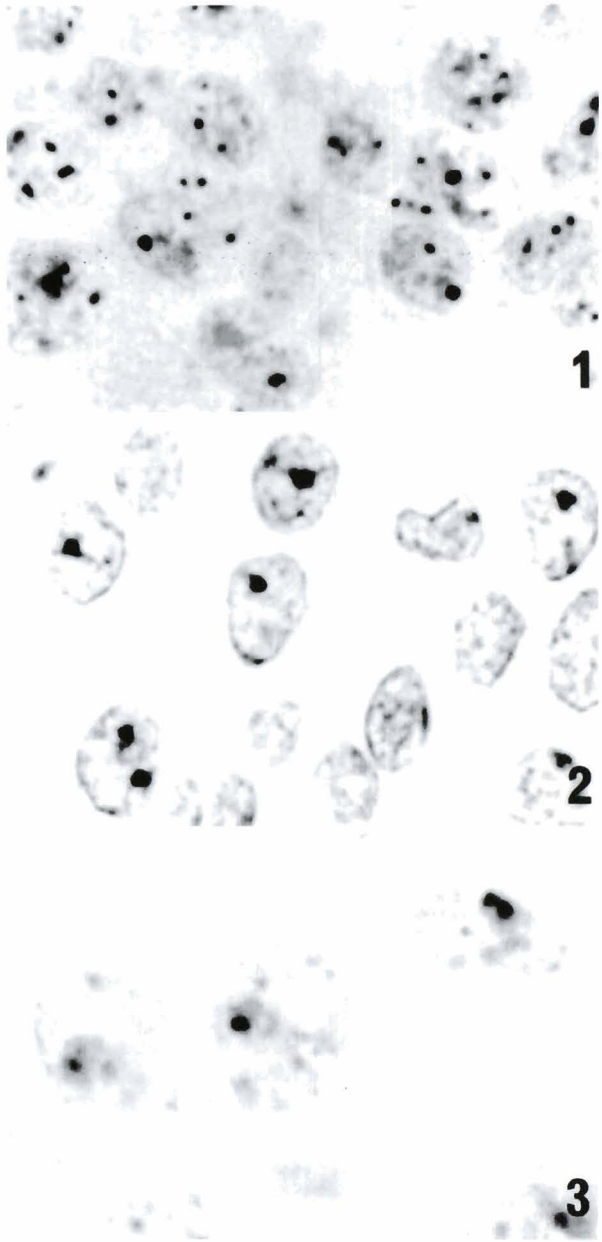
In the samples from all the groups analyzed a good reaction with silver colloid was visualized, with the observation of well-defined black silver-stained nuclear dots. The nuclei were stained with a gentle non-specific reaction, yellowish in colour, such that it was not necessary to apply any counterstaining to identify the nuclear contour.

Direct examination with the light microscope revealed that in samples from the younger animals silver-stained particles were scattered throughout the nucleus and that in these animals the number of particles per nucleus was greater than in the older ones (Figs. 1, 2). Clusters of aggregated particles were frequently seen in samples from 28- and 80-day-old animals (Fig. 3).

Numerical data obtained after image processing and quantification of nuclear areas and AgNOR-related parameters are shown in Table 1. The morphometric study showed an increase of nuclear area and mean size of silver-stained particles with age. Inversely, the mean area and numbers of AgNORs per nucleus progressively decreased from 1- to 80-day-old animals (Fig. 4).

Discussion

In recent years, several studies have related the expression of AgNORs with cellular proliferation. Accordingly, a linear relationship between the mean numbers of AgNORs per nucleus and both the percentage of S-phase cells (Crocker et al., 1988) and



Figs. 1. to 3. Silver-stained organizer regions (AgNORs) in cell of the rat anterior pituitary. Note the different nuclear size on AgNOR expression between samples from 1- (**Fig. 1**) and 80-day-old rats (**Fig. 2**). Aggregation of silver stained particles (arrow in **Fig. 3**) is frequently seen in samples from 80-day-old animals. Figs. 1 and 2, x 8,300; Fig. 3, x 13,000)

Table 1. AgNOR-derived parameters (mean±SEM) obtained from cells of the pituitary of rats at different ages. Significantly different ($p<0.05$, Fisher test) from data of (1) 1-, (2) 7- and (3) 14-day-old ras, in the same column.

AGE (DAYS)	NUCLEAR AREA	TOTAL AREA OF AgNORs PER NUCLEUS	NUMBERS OF AgNORs PER NUCLEUS	MEAN AgNOR PARTICLE AREA
1	26.08±0.60	1.96±0.11	4.10±0.22	0.49±0.02
7	25.38±0.56	1.92±0.13	4.08±0.28	0.53±0.06
14	31.35±0.63[1,2]	1.76±0.09	3.51±0.27	0.57±0.04
28	31.25±0.82[1,2]	1.69±0.09[1]	2.94±0.23[1,2]	0.59±0.04
80	31.42±0.76[1,2]	1.52±0.08[1,2]	2.43±0.22[1,2,3]	0.63±0.07[1]

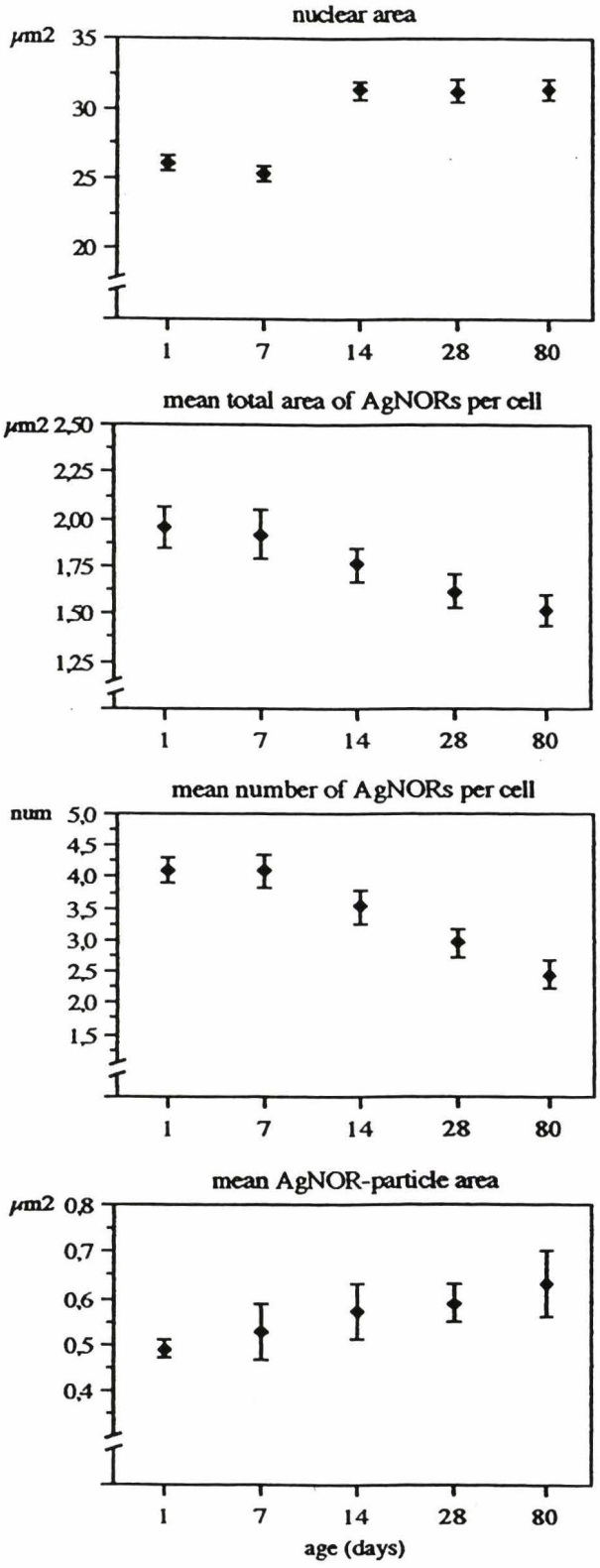


Fig. 4. Graphic representation of morphometric data corresponding to nuclear area and AgNOR-derived parameters from rat anterior pituitary.

the proportion of cells that have nuclear Ki67 immunoreactivity (Hall et al., 1988) has been reported in non Hodgkin's lymphomas. Similarly, a good correlation between AgNOR expression and the growth rate, as assessed by (3-H)-thymidine incorporation (Derenzini et al., 1989, 1990) and calculation of the doubling time (Derenzini et al., 1989) has been documented in several tumour cell lines. More recently, a progressive increase in the expression of AgNORs through the cell cycle has been reported when studying rat thymic cells sorted at different phases of the cell cycle according to DNA content (Carbajo et al., 1991; Orfao et al., 1992). All these data support the notion that AgNOR expression can be considered as a marker of cellular proliferation and that the expression of AgNORs is linked to DNA synthesis.

In the present study, a decrease in the mean area and numbers of AgNORs has been found to occur during postnatal development in cells of the rat anterior pituitary. If, as stated above, a direct relationship between AgNOR expression and proliferative activity is to be assumed, our results should be interpreted as a signal of a progressive reduction in the proliferative activity of the anterior pituitary during growth. This, indeed, would fit the marked decline of cellular proliferation previously reported to occur in this gland during postnatal development (Carbajo-Pérez et al., 1989; Carbajo-Pérez and Watanabe, 1990). However, this relation must be carefully considered.

The overall proliferative activity of the pars distalis of the anterior pituitary decreases from 6.4% at 2 days to 1.7% at 30 days (Carbajo-Pérez and Watanabe, 1990), and at 60 days it is only 0.5% (Carbajo-Pérez et al., 1989). Although the decrease in the proliferative activity throughout the postnatal period is significant, there are no large differences in the absolute values of proliferating cells during this period. If an increased expression of AgNORs in a sample is related to the number of proliferating cells it seems improbable that the variations found in the expression of AgNORs in the present study would be due exclusively to proliferative activity. In a sample of 100 cells, such as that used here, taken from the anterior pituitary of newborn rats, no more than six or seven proliferating cells would be included. Similarly, no more than one or two proliferating cells would be found in a sample taken from 30-day-old animals. Hence, it is difficult to assume that expression of AgNORs in a sample of a hundred cells is determined by such a small proportion of proliferating cells and, furthermore, it is not easy to admit that just five cells are responsible for the different AgNOR expression found in 1- and 28-day-old animals.

Expression of AgNORs is not only related to cellular proliferation, it is also thought to reflect cell differentiation. In this sense, the existence of a reduction in the expression of AgNORs (Smetana and Likovski, 1984) and a decrease in the number and volume of fibrilar centres (Zatsepina et al., 1988a) has been described in advanced maturation stages of erythroid

cells compared to earlier phases of development. Yan and Stanley (1988) reported that treatment on a melanoma cell line with retinoic acid induced morphological differentiation concomitant with a significant decrease in AgNOR activity. More recently, Edwards et al. (1991) have shown that AgNOR numbers are intimately related to differentiation state in U937 cell lines.

Differentiation and maturation of the different types of cells of the anterior pituitary starts by the 15th day of foetal life (Nemeskeri et al., 1976; Watanabe and Daikoku, 1979; Simmons et al., 1990) and continues during the postnatal period (Childs et al., 1981; Hoeffler et al., 1985). The important changes in the proportion of the various cell types of the anterior pituitary that take place during the first two weeks of postnatal life, which suggest an increase in the actual numbers of these cells, are not accompanied by parallel changes in the proliferative activity of the different cell types. This points to a major role of differentiation, rather than division of already differentiated cells, in the acomplishment of the cytological structure of the anterior pituitary (Carbajo-Pérez and Watanabe, 1990). Ultrastructural features of anterior pituitary cells may also serve as an index of postnatal maturation. Accordingly, a gradual increase in the development of cell organelles and secretory granules occurs through the 3rd to the 6th postnatal days with a spurt of activity between the 6th and 11th postnatal days. A further increase seems to take place on the 20th day after birth (Fink and Smith, 1971).

It is well known that immature forms of anterior pituitary cells are smaller in size than mature cell types (Childs et al., 1981) and this also applies to nuclear area (Rubio et al., 1989). Thus, the greater nuclear area found in our study from the 14th postnatal day onwards is in good agreement with the above-mentioned data on development of the anterior pituitary.

The maturation process that takes place in the anterior pituitary during the postnatal period might well be the cause of the progressive decline in the expression of AgNORs here reported.

Classically, the mean number and/or mean area of AgNORs per nucleus have been used to evaluate AgNOR expression. This was also done here. It should be noted that the changes in AgNOR numbers and mean AgNOR particle area should be interpreted bearing in mind that aggregates of silver-stained particles are considered as units in our analysis, as has already been done by other researchers (Giri et al., 1989). Using this approach for quantification of the AgNOR reaction, the actual size of individual AgNOR particles is not obtained since the area of several particles gathered to form a cluster is registered as one. The apparently paradoxical increase in mean AgNOR particle size seen in this report may well correspond to aggregation phenomena characteristic of the older animals of our series. Aggregation phenomena of AgNOR particles may also be related to maturation since fusion of individual fibrilar centres is the expression of partial inactivation of ribosomal genes, as shown by Zatsepina et al. (1988b) in interphase pig embryo kidney cells.

The functional bases of the correlation between AgNOR expression and either cellular proliferation or cell differentiation are not yet clear. So far, the relation between AgNORs and either of both variables is rather empirical. Comparison of our data on AgNOR expression with previously reported data on the proliferative activity and cell maturation and differentiation of anterior pituitary cells during a similar period lead us to conclude that, at least in our experimental model, variations in the expression of AgNORs are more related to phenomena of cellular maturation than to proliferative activity. This report gives further support to the notion that though cellular proliferation is an important factor conditioning the expression of AgNORs, other variables, such as cellular differentiation, should be taken into account when studying tissues during development or neoplasms.

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