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Image Analysis of Argyrophil Nucleolar Organizer Regions (AgNORs) in Oestrogen-Induced Rat Anterior Pituitary Hyperplasia: Comparison of Automatized (LUCIA M) and Non-Automatized Evaluation

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Summary

Argyrophil nucleolar organizer regions (AgNORs) were morphometrically evaluated in rat anterior pituitaries of control and oestrogenized rats using an automatized (LUCIA M – Laboratory Universal Computer Image Analysis Micro) and a non-automatized approaches to investigate not only the sensitivity and effectiveness of the automatized procedure but also the morphology of AgNORs in oestrogen-induced rat anterior pituitary hyperplasia. In the experimental oestrogen-induced rat anterior pituitary hyperplasia, the image analysis system LUCIA M was compared to a non-automatized morphometric procedure and proved to be very efficient and yielded analogical results. The AgNOR morphology in the oestrogenized pituitaries was characterized by an increase of the total AgNOR area and clustering of AgNORs in the large nucleoli.

Key words

AgNORs – Morphometry – Computer image analysis – Oestrogen – Rat anterior pituitary

Introduction

The adenohypophysis is a target tissue for oestrogens and its growth can be experimentally induced in rats by oestrogen treatment (Selye 1949). In addition, adenohypophyseal tumors can be produced by prolonged oestrogen treatment (Waelbroeck-Van Gaver 1969a,b).

Using light and electron microscopy, we studied the changes of oestrogenized adenohypophysis (Dušková and Schreiber 1987, 1989) that were morphologically characterized by an increased number and activity of prolactin cells at the light microscopy level and by rather striking ultrastructural changes, especially by nucleolar activation (increased number and size of nucleoli determined by the non-automatized point counting method and presence of lacy nucleoli with visible nucleolonemata).

Recently, considerable attention has been paid to the argyrophil nucleolar organizer regions (AgNORs), loops of ribosomal RNA and associated argyrophil proteins located on five pairs of human chromosomes. Their amount and morphology reflect the ploidy as well as cellular proliferation and transcription activity (Giri *et al.* 1989, Jan-Mohamed *et al.* 1989, Suresh *et al.* 1990, Jordan 1991, Leek *et al.* 1991) and is therefore used as a predictor of tumor progression. Nevertheless, the approaches to AgNOR evaluation and quantification have not yet been unified, irrespective of the current effort for automatization and standardization of this tedious procedure (Baak *et al.* 1994, Crocker *et al.* 1989, Derenzini and Treré 1991, Rüschoff *et al.* 1990).

We have developed a macro (a constant series of computer program commands) for automatic measurement of AgNORs in the colour image analysis system LUCIA-M (Laboratory Universal Computer Image Analysis Micro, Laboratory Imaging, Prague) (Dušková and Povýšil 1995). The aim of this study was to compare the results of the non-automatized and automatized AgNOR evaluations and to test the effectiveness and sensibility of the automatized system using the known model of oestrogen-induced anterior pituitary hyperplasia. At the same time we also intended to learn more about the AgNOR dynamics in the hyperplastic process.

Material and Methods

Material

Rat adenohypophyses (Wistar rats VELAZ, Prague) from 10 control and 10 oestrogenized rats were used (for details see Dušková and Schreiber 1989). The pituitaries from all twenty animals were investigated using light and electron microscopy. Three pituitaries from each group were randomly chosen and cut in several planes for morphometric evaluation. At least 150 cells in each specimen were measured.

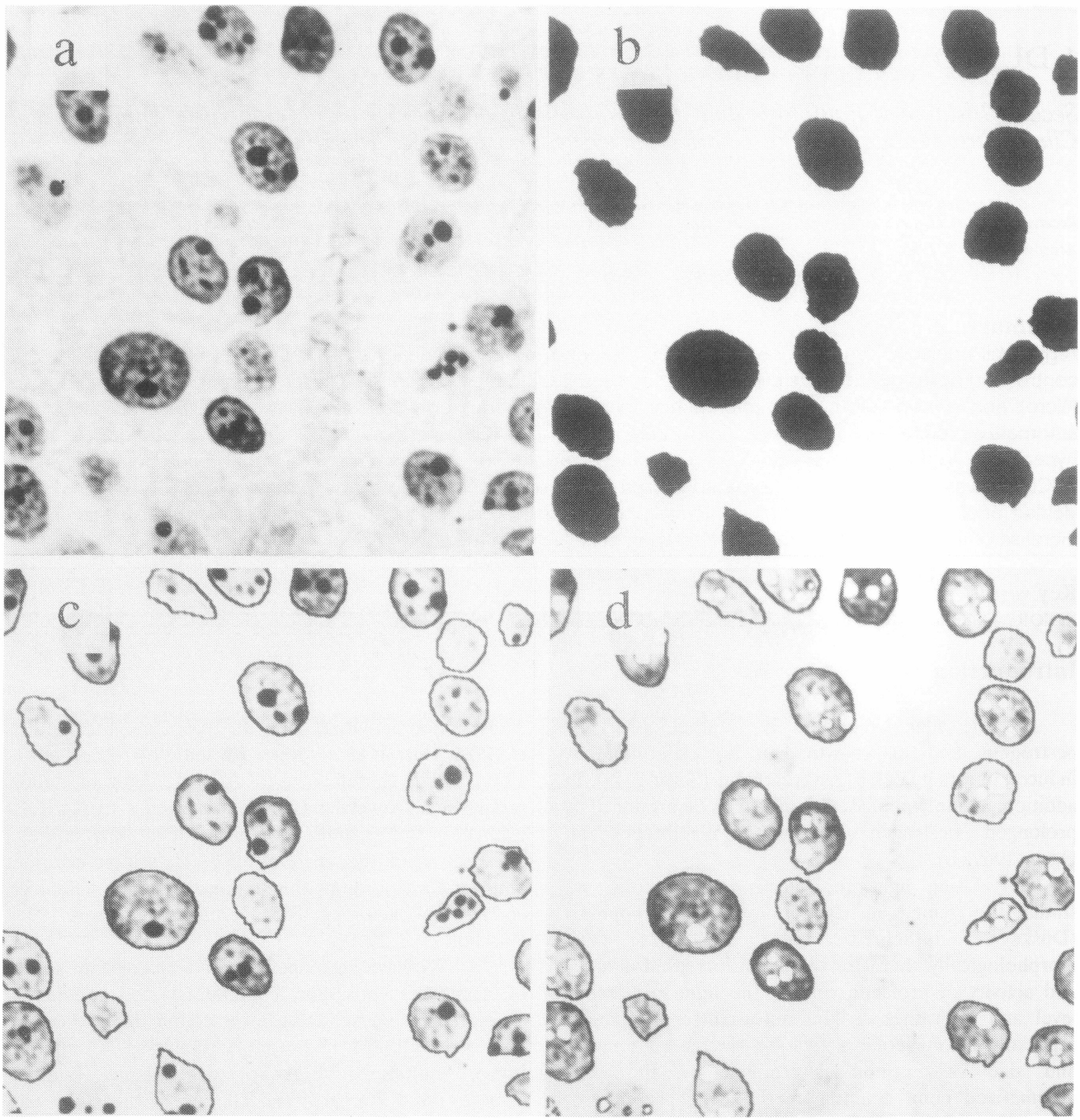

Fig. 1
AgNOR impregnation in the anterior pituitary cells of oestrogenized rat (a), the same field after the nuclei had been thresholded (b), the same field, nuclei contoured (c), and the same field, nuclei contoured and AgNORs thresholded (white spots) (d). In the subsequent evaluation the nuclei containing either no AgNORs or extranuclear AgNOR-simulating silver precipitates were excluded from the measurement. Enlarged 1400 x.

Staining

Three to four micrometer sections from formalin-fixed paraffine-embedded adenohypophyses were impregnated according to Rüschoff *et al.* (1990) with an impregnation time of 25–30 min that allowed us to distinguish the granules (AgNORs) inside one nucleolus while focusing to a depth of one micrometer.

Hardware

PC 486/66 MHz, 16 MB RAM, Graphic Adapter VGA Video Seven, hard disc 340 MB, MS mouse, Frame grabber, TV camera with RGB signal (JVC TK 1070 E), microscope Nikon Microphot FXA.

Software

MS DOS 6.0, Lucia M 2.991, MS Windows 3.1, Excel 4.0.

The automatized measurement process (A)

An introductory macro includes microscope adjustment (8 V, ND8, obj. 40x/0.8, additional enlargement 2x), shading correction, selection of the features measured, calibration and start of the measuring macro, that is restarted unless discontinued by the user measuring the objects in selected fields, as described in detail previously (Dušková and Povýšil 1995). Briefly, the measurement is introduced with the picture quality interactive optimalization (contrast, smoothing). After the picture has been captured the user interactively completes predefined thresholding (object detection) and edits the binary picture that has been filtered automatically by the selected restrictions. The nuclei binary picture is saved in the memory and its contours are used to define the nuclei in which an analogous process is performed with AgNORs. Binary operations ensure the relation of the nucleoli and AgNORs – only nuclei containing at least one AgNOR in the plane sectioned were measured and no artificial precipitates lying outside the nucleoli were evaluated (Fig. 1).

The number of AgNORs per one nucleus was determined using advanced morphology – homotopic marking. Intranucleolar clusters of AgNORs were mostly counted together by the automatized system.

The following features were measured in 150 cells in randomly selected fields: nuclei – area, equivalent diameter (i.e. diameter of a circle having the same area as the object measured), nucleoli – area, equivalent diameter, AgNORs – total number per nucleus section (indiscernible in the nucleolus were counted as one).

The data obtained were exported and evaluated in the statistical program Excel 4.O.

Non-automatized procedure (NA)

Fifty cells in randomly selected fields were evaluated with an ocular micrometer for the following features: nuclei – maximal diameter, nucleoli – number, maximal diameter of the largest, AgNORs – total number of extra- and intranucleolar dots (focusing during counting through one micrometer depth of the section)

Typing of the nucleoli was performed according to the scheme of Hansen and Andersen (1992) (Fig. 2).

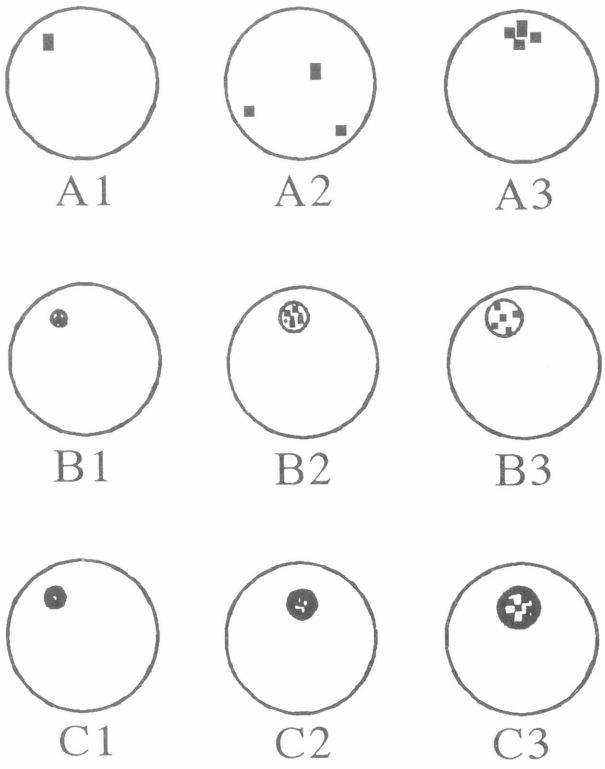

Fig. 2
AgNOR typing – satellite AgNORs: single A1, scattered A2, grouped A3, fine granular AgNORs in nucleoli: small B1, medium B2, large B3, coarse to solid AgNORs in nucleoli: small C1, medium C2, large C3.

The non-automatized procedure was performed using the Zeiss NU-2 microscope, immersion objective 100x/1.3, final enlargement 100x. The other conditions were identical with the automatized procedure.

Statistics

The significance of the differences between groups with pooled data from the three randomly selected animals was tested using the Wilcoxon (Mann-Whitney) test.

Table 1
Nuclear and AgNOR areas and equivalent diameters in control and oestrogenized rats

Rat	MNA	MED	MAgNORA	MAgNORED
C1	18.08 ± 4.01	4.77 ± 0.53	0.98 ± 0.52	1.07 ± 0.31
C2	18.14 ± 4.21	4.77 ± 0.56	1.03 ± 0.59	1.10 ± 0.33
C3	20.07 ± 4.79	5.02 ± 0.60	1.05 ± 0.61	1.11 ± 0.33
E1	28.25 ± 10.36	5.90 ± 1.09	2.11 ± 1.52	1.53 ± 0.59
E2	24.91 ± 8.72	5.55 ± 0.93	1.35 ± 0.91	1.23 ± 0.45
E3	23.27 ± 7.56	5.37 ± 0.86	1.83 ± 1.31	1.42 ± 0.56

Data are means ± S.D. C1, C2, C3 – control, rats, E1, E2, E3 – oestrogenized rats, MNA – mean nuclear area, MED – mean equivalent diameter, MAgNORA – mean AgNOR area, MAgNORED – mean AgNOR equivalent diameter

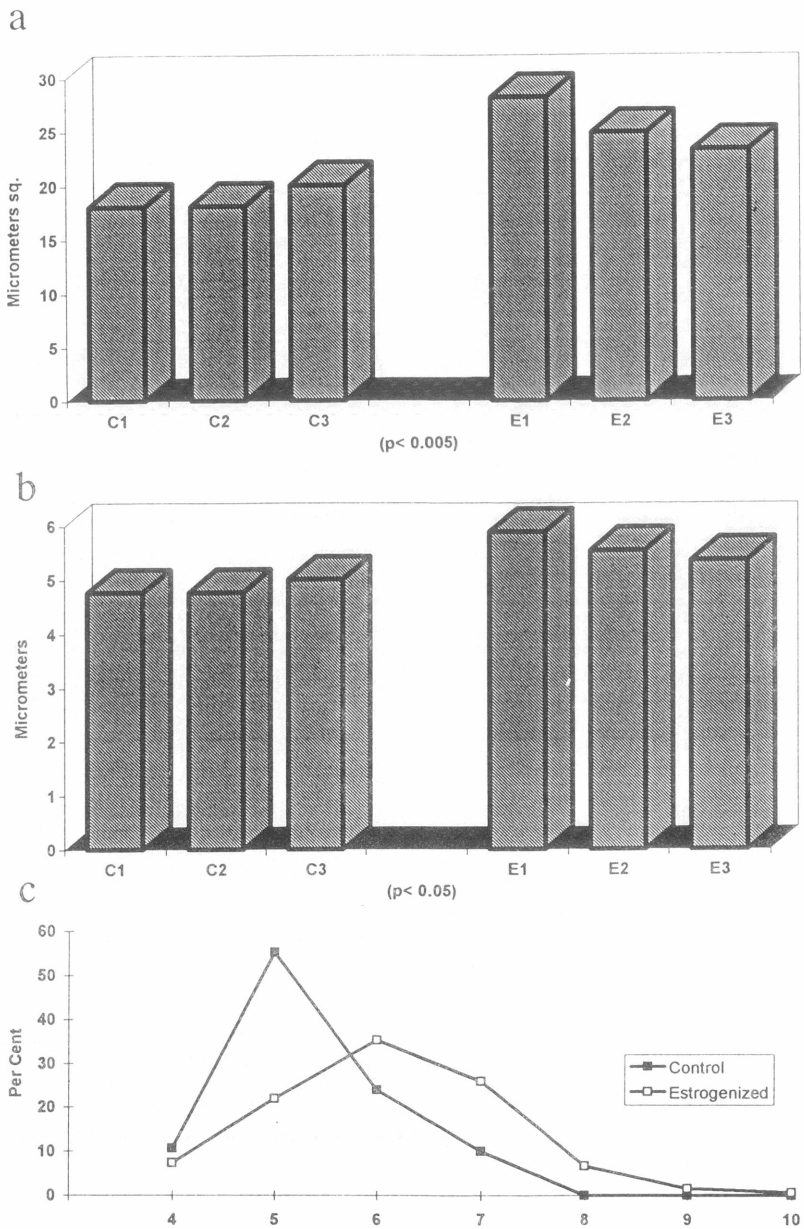

Fig. 3
Mean nuclear area (a), mean equivalent diameter (b) and distribution of maximum nuclear diameter determined by non-automatized procedure (c) of rat anterior pituitary cells. C1, C2, C3 – control rats, E1, E2, E3 – oestrogenized rats.

Results

Both non-automatized (NA) and automatized (A) measurement procedures demonstrated nuclear hyperplasia in oestrogenized rats (Table 1, Figs 3a,b,c) as expressed by means of mean maximal diameter (NA), mean equivalent diameter (A) and nuclear area (A). The difference was highly significant ($p<0.005$). The same significance was true for the enlarged nucleoli (Figs 4a,b,c) though the values measured with the automatized system were modified by counting and measuring individual extranucleolar dots.

No significant difference was found between the controls and oestrogenized rats as far as the number of AgNORs per nucleus in the (A) procedure is concerned. The (NA) procedure which takes into account intranuclear granules, indicated a highly significant increase of their total intra- and extranucleolar numbers in the oestrogenized animals. (Figs 5a,b).

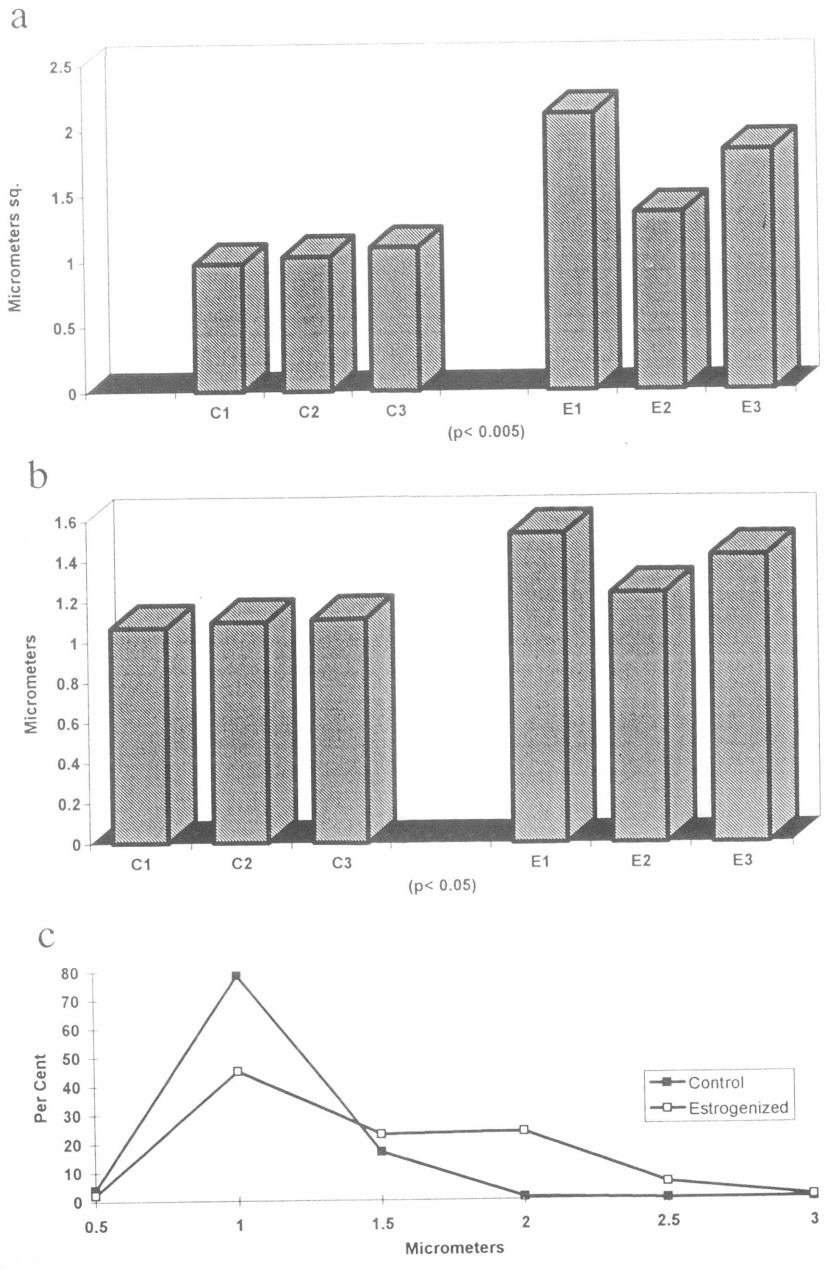

Fig. 4
Mean AgNOR area (a), mean AgNOR equivalent diameter (b) and distribution of maximum nuclear diameter determined by non-automatized procedure (c) of the rat anterior pituitary cells. C1, C2, C3 – control rats, E1, E2, E3 – oestrogenized rats.

In classifying the nucleoli according to Hansen and Andersen, the most important was the increase in the number of large nucleoli with fine granular AgNORs inside, i.e. nucleolus type B3 (Fig. 6).

The times needed for (NA) and (A) procedures were one hour and 15 minutes per slide respectively, not taking into account the fact, that this time included statistical evaluation available in (A), while the statistical evaluation in (NA) needed additional time. This resulted in a difference of approximately 7–10 times in favour of (A).

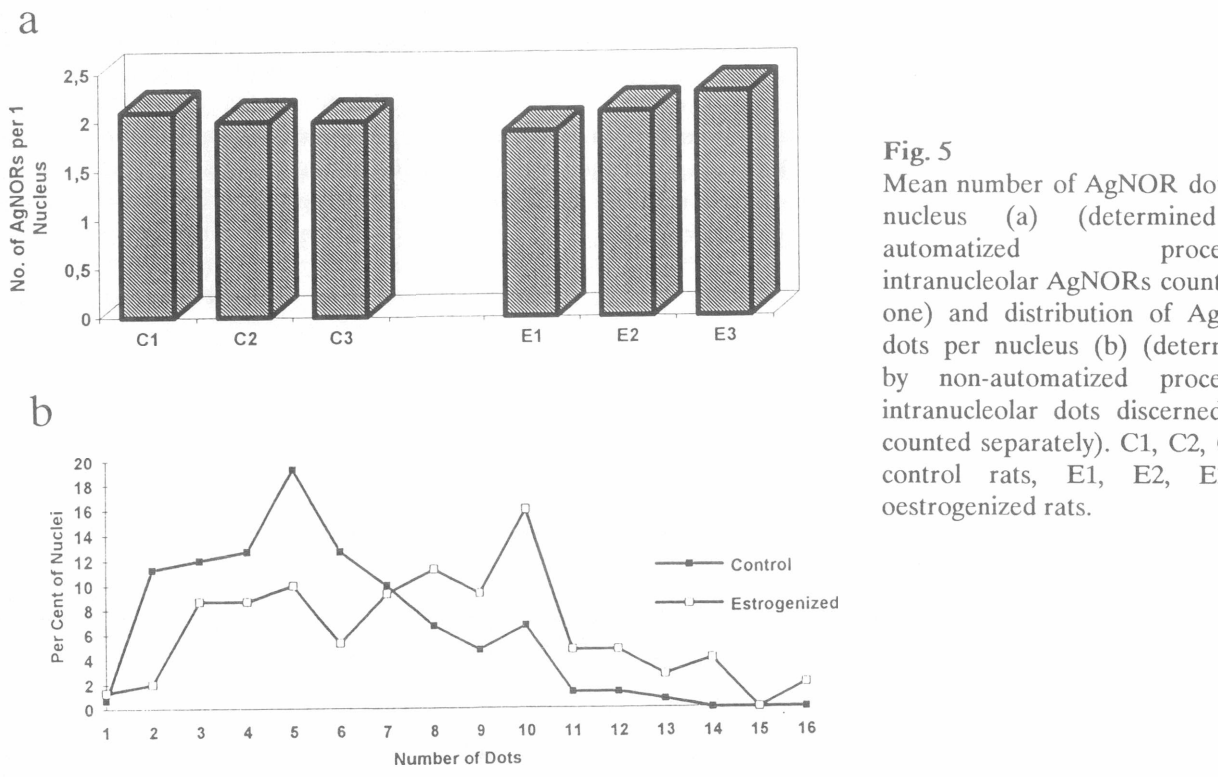

Fig. 5
Mean number of AgNOR dots per nucleus (a) (determined by automatized procedure, intranucleolar AgNORs counted as one) and distribution of AgNOR dots per nucleus (b) (determined by non-automatized procedure, intranucleolar dots discerned and counted separately). C1, C2, C3 – control rats, E1, E2, E3 – oestrogenized rats.

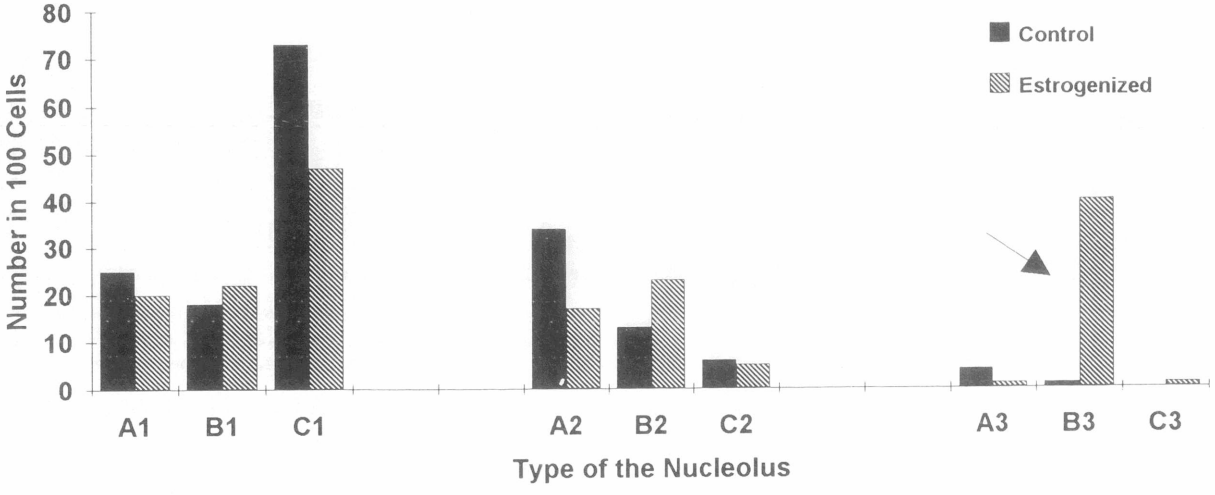

Fig. 6
Typing of the nucleoli in 100 anterior pituitary cells. A1...C3 types of the nucleolus (see Fig. 2).

Discussion

The effect of oestrogen on anterior pituitary cells has been known for many years. Prolonged oestrogenization is able to elicit transplantable tumours and even carcinomas after hyperplasia and functional activation (Waelbroeck-Van Gaver 1969a,b).

Structural and ultrastructural changes of pituitary cells connected with activation appear from the initial stages of oestrogen-induced pituitary hyperplasia. They are accompanied by chromosomal changes and it is therefore not surprising that AgNOR changes are also present. Using non-automatized ultrastructural morphometry (Dušková and Schreiber 1987, 1989), we have found striking enlargement and

activation of nucleoli in the anterior pituitaries of rats oestrogenized for three weeks. A scavenger of free radicals, methylene blue, inhibits the adenohypophyseal growth after oestradiol treatment (Schreiber *et al.* 1993) as well as the increase of one of the acute phase proteins, ceruloplasmin (Maruna *et al.* 1994). Since AgNORs reflect the adenohypophyseal oestrogen induced growth stimulation, in which the increased blood ceruloplasmin level plays an undetermined role, the relations of these findings deserve further studies. Having evaluated the AgNORs in a pilot study in human thyroid follicular lesions using the non-automatized procedure (Dušková 1992) and having thus obtained our own experience with the laboriousness of such an evaluation, we decided to use an automatized image analysis system (LUCIA M, Laboratory Imaging, Prague) for further studies and applications. Our effort was focused on the development of a sensitive and effective tool (macro with the possibility of user's interactive approach) for AgNOR evaluation (Dušková and Povýšil 1995). Its possibilities were tested on an experimental model of rat oestrogen pituitary hyperplasia used in our laboratories.

A comparison of the results obtained by our selected model with an ocular grid and by the automatized system proved the high sensitivity of the latter in describing the features of hyperplasia with the exception of the total number of intra- and extranucleolar dots. The picture displayed and evaluated in the computer cannot usually discern individual organizers inside the nucleolus. Two or three AgNORs can be discerned as a maximum while in the NA procedure by focusing through the entire thickness of the nucleus the number of organizers inside as well as outside the nucleolus are considerably higher. Our comparison of (A) and (NA) procedures shows that this feature may be partly substituted for total absolute or relative AgNOR area. Nevertheless, studies on nucleolar kinetics (Smetana *et al.* 1984, 1987, 1990) as well as on the structure of AgNORs in cells of various maturity (Grotto *et al.* 1991) or in experimental carcinogenesis studies (Carbonelli *et al.* 1994) stress the information value of AgNOR structure. We have observed a shift towards large nucleoli with many finely dispersed dots together with an increase of the AgNOR area in agreement with the results on prostatic hyperplasia (Hansen and Ostergard 1990). Practically no nucleoli with coarse AgNORs were present.

Thus the non-automatized approach may provide valuable information. On the other hand, non-automatized evaluation is a vulnerable procedure burdened with rather high interindividual and personal error. Even if enough experience is gained to lower this kind of mistakes, the procedure remains so time-consuming that it has no prospects of being used routinely.

The large number of papers dealing with AgNOR evaluation may be divided into two groups. The first includes those using non-automatized evaluation, mostly only counting without measurement. An immersion lens with magnification of one hundred and gradual focusing is used to discern and count the separate AgNORs in the nucleolus. One to two hundred cells are usually evaluated. The second group of investigators is trying to computerize the evaluation of AgNORs and to look for the most sensitive and informative descriptors of this intranuclear structure.

Our effort to develop a sensitive tool for AgNOR analysis in our newly introduced image analysis system LUCIA M is oriented towards the intended routine improvement of tumour diagnostics. It has been proven for many nosological entities that AgNORs are useful predictors of tumour behaviour (Aubele *et al.* 1994, Baak *et al.* 1994, Giri *et al.* 1989, Hansen and Ostergard 1990, Ishida *et al.* 1993, Jan-Mohamed *et al.* 1989, Korkolopoulou et al 1993, Lim *et al.* 1992, Mourad *et al.* 1993, Rüschoff *et al.* 1993a,b, Salmon and Kiss 1993, Toikkanen and Joensuu 1993). These practical applications and correlations with other proliferation markers have been employed using both approaches and many various AgNOR descriptors. It is well known that the total amount of AgNORs reflects an increasing ploidy and proliferation activity in a variable ratio (Carbonelli *et al.* 1994, Jan-Mohamed *et al.* 1989, Suresh *et al.* 1990). For estimating tumour prognosis these two effects need to be strictly differentiated as both increasing ploidy and proliferation contribute to the undesirable behaviour of tumours.

Using a special more interactive approach, the dispersion of AgNORs (AgNOR distribution) inside the nucleolus can also be evaluated by the automatized system (Rüschoff *et al.* 1993a,b). This type of evaluation requires more time and effort because it represents a compromise of automatized and non-automatized evaluation. Providing that a very well standardized system of staining and internal and external controls is employed, it may give reproducible results and valuable information. In our system tested (LUCIA M), such an approach would be feasible if it appears to be necessary for practical applications.

We conclude that in experimental oestrogen-induced rat anterior pituitary hyperplasia, the image analysis system (LUCIA M), tested and compared to a non-automatized morphometric procedure yielded analogous results. The AgNOR morphology in the oestrogenized pituitaries was characterized by an increase of the total AgNOR area and clustering of AgNORs in the large nucleoli.

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