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In vitro modifications in the proliferation rate of prolactin cells are accompanied by nuclear morphometric variations

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Summary. In order to establish the correlation between in vitro proliferation rate and morphometric variations of prolactin immunoreactive cells, a morphometric study was carried out in rat pituitary monolayer cultures by means of the double immunocytochemical staining methods employing mouse monoclonal antiproliferative cell nuclear antigen (PCNA) and rabbit anti-prolactin (PRL) as primary antibodies. PCNA was found to be an adequeate marker for proliferation in pituitary monolayer cultures. 48.35±2.78% of the cells present in the culture were in active cell cycle after 3 days of incubation and a similar proportion, 54.93±2.83% was found after 7 days. On the 3rd day, PRL immunopositive cells accounted for 15.16±0.21% of the total cellular content in the dishes and 8.68±0.12% of the PCNA immunoreactive cells were also PRL immunopositive cells and, 60.95±2.65% of PRL cells stained for PRL and PCNA. On the 7th day, an increase to 32.18±0.60% of PRL cells was found; the PCNA and PRL cells accounted for 60.32±2.34% of the total PRL cells, and 19.88±1.09% of the PCNA reactive cells stained for PRL. Additionally, the morphometric analysis performed after 3 or 7 days of incubation showed that, while the size of PRL cells remained unmodified, the nuclear area had increased on the 7th day in relation to the 3rd day ($p<0.01$). These results suggest: 1) PCNA is a valid proliferative marker for pituitary cells in cultures; 2) a very high percentage of the PRL cells was in early proliferation; 3) on the 7th day of incubation, the proliferative rate of PRL cells was very similar to that observed on the 3rd day, suggesting a maintained proliferation for PRL cells at early incubation phases; and 4) the cellular activity, expressed as variations in the nuclear size, was higher on the 7th day than on the 3rd day; in addition, the numerical density of PRL cells increased.

Key words: Cell cycle, Prolactin, PCNA, Immunocytochemistry, Morphometry

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Introduction

In in vitro conditions, PRL cells are usually stimulated, principally because monolayer cultures are not inhibited by hypothalamic factors like dopamine (MacLeod, 1976; Weiner and Ganong, 1978) and because they are intermingled with pituitary VIP-producing cells (Segerson et al., 1989; Lam et al., 1990; Carretero et al., 1992a).

PCNA is a nuclear protein associated with DNA synthesis, being synthesized during G1-S-G2 phases of the cell cycle (Mathews et al., 1984; Tan et al., 1986; Prelich and Stillman, 1988). Studies on various tissues, including neoplasms, as well as cell cultures, have shown that the immunocytochemical detection of nuclear PCNA expression correlates with data obtained by other methods employed to evaluate cellular proliferation (García et al., 1989; Battersby and Anderson, 1990; Dawson et al., 1990; Hall et al., 1990; Levison et al., 1990; Woods et al., 1990; Coltrera and Gown, 1991; Scott et al., 1991; van Dierendonck et al., 1991; Wijsman et al., 1992). PCNA has been reported to be more specific than other methods for determining the in vivo proliferative activity of rat pituitary cells (Oishi et al., 1993).

In order to establish the proliferation rate of PRL immunopositive cells under in vitro conditions, and their correlation with morphometric variations in different incubation periods, a study using double immunohistochemical staining was carried out for PCNA and PRL on the 3rd and 7th days of incubation, determining the variations of cytometric parameters, the proliferative rate of PRL cells and the percentage of both PCNA- and PRL-immunoreactive cells.

Materials and methods

Pituitary cultures

Following anaesthesia with isoflurane, male Wistar rats (175-200 g) were killed by decapitation and the anterior pituitary glands were removed and washed in Earle's balanced salt solution. Enzymatic dispersion was

carried out by incubation for 15 minutes at 37 °C in Hank's solution to which 0.15% $MgCl_2$, 0.1% papain, 0.01% DNase and 0.1% of neutral protease had been added. Mechanical dispersion was achieved by passing the pituitaries through Pasteur pipettes and 20- to 22-gauge needles. After centrifugation, the supernatant was removed and the cells were resuspended in an appropriate volume of Dulbecco's modified Eagle's medium, supplemented with 10% calf serum, 5% foetal calf serum, 10% L-glutamine, 1000 IU/ml of penicillin and 1000 IU/ml of streptomycine (Carretero et al., 1991a; Sánchez et al., 1991). The cells were seeded on 20 culture dishes (30x15 mm) at a final concentration of $5x10^5$ cells/dish and incubated at 37 °C in a 5% CO_2/95% air atmosphere for 3 or 7 days. The dishes were divided into two groups: one of them was analyzed on the 3rd of incubation, the media of the second group of dishes were replaced on the 4th day and the dishes analyzed on the 7th day of incubation (10 dishes per group were used). At the end of the incubation period, the culture medium was removed, the dishes were carefully washed with Dulbecco's sterile PBS, and the cells were fixed in 4% paraformaldehyde and 15% picric acid in phosphate buffer (0.1M, pH 7.4) for 1 hour, followed by careful rinsing in PBS.

Immunocytochemistry

After blocking endogenous peroxidase with methanol and H_2O_2 and non specific reactions of the secondary antibody by incubation in normal goat serum (1:30) in TBS (Tris-saline buffer 0.05M, pH 7.6 in 0.8% of NaCl and 0.1% of Triton X-100) the cells were incubated overnight at 4 °C with the mouse PC10 mAb (Dako, lot. 21 diluted 1:3000 in TBS). Biotinylated goat anti-mouse IgG (Dako, lot. 061 diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit, Dako lot. 081diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. The reaction was developed in freshly prepared 3-3'DAB (0.25% in TRIS buffer containing 0.03% H_2O_2). Following the PCNA immunolabelling, the peroxidase-antiperoxidase (PAP) reaction was performed for the detection of PRL, by overnight incubation with rabbit antiserum against PRL (Dako lot. 042a, diluted 1:1000) at 4 °C. Swine anti-rabbit IgG (Dako lot. 022 diluted 1:200) and rabbit PAP soluble complex (Dako lot. 021, diluted 1:200) were successively applied at room temperature for 30 minutes. The reaction was developed using 4-chloro-1-naphtol ($1.7x10^{-3}$M in 3% absolute ethanol and TRIS-buffer containing 0.3% H_2O_2). Method controls included substitution of the primary antibodies with normal, mouse or rabbit, serum or TBS, as well as omission of the secondary antibody or ABC and PAP complex. Specificity of swine anti-rabbit serum was tested by substitution and preabsorption test with mouse IgG. After both tests, no immunoreactivity was detected. The cross-reaction with immunoglobulins of mouse or rat was determined by ELISA and it was also very low, less than 1%.

Morphometry

Using a MIP-2 (IMCO 10) image analyzer, the cellular, nuclear and cytoplasmic areas of 50 PRL immunoreactive cells per dish (500 cells per group), randomly chosen, were calculated in an interactive fashion.

Quantization

Forty fields per dish were evaluated using an Axiophot Zeiss microscope equipped with an ocular grid at a final magnification of x400. The fields were ramdoly selected from different areas of the dishes: the surface of each dish was subdivided into 8 areas, and 5 fields per area were evaluated. In each field the following parameters were determined: 1) The total number of cells; 2) PCNA-positive cells; 3) PRL-positive cells (PRL-index, «PRL-I»); and 4) PCNA-positive cells/PRL-positive cells (PRL-PCNA labelling index, «PRL-PCNA-I»); and 4) PCNA-positive cells/PRL-positive cells (PRL-PCNA labelling index, «PRL-PCNA-I»); 2, 3 and 4 were calculated as the percentages from the total number of cells per field; the percentage of proliferating PRL cells from the PRL-positive cells, labelled by PCNA and PRL jointly, was calculated (% PRL-PCNA) from 4,000 cells per dish.

Statistical analysis

The results obtained were processed statistically and the differences observed were compared using analysis of variance, accepting as significant values of $p<0.05$ for the Fisher-PLSD and Scheffé F tests jointly.

Results

Analysis of PRL and PCNA immunopositive cells

PCNA immunopositive cells were identified as brown labelled cells and they were scarcely distributed on the surface of the dishes, but clusters of immuno-positive cells were frequently observed (Fig. 1).

PCNA staining was found almost exclusively in the nuclei, except for 2.51% of the cells which showed cytoplasmic reaction. Different intensities of reaction were observed; several cells showed strongly-labelled nuclei (thick arrow in Fig. 1), while the other cells had weak nuclear immunoreaction (thin arrows in Fig. 1). No differences in pattern or intensities of immunoreaction for PCNA were found after 3 or 7 days of incubation.

Table 1 shows the percentage of PCNA cells on the 3rd day in relation to the total number of cells. This percentage increased, but it was not significantly modified on the 7th day.

PRL-cells were identified as dark blue-stained cells. The blue reaction for prolactin was found only in the

cytoplasm of positive or negative PCNA cells. No differences in pattern and intensity of immunoreaction were found related to the time of incubation (Figs. 2, 3).

The PRL-index was higher on the 7th day than on the 3rd day. On the 3rd day, the percentage of PRL cells was 15.16±0.21%; however, on the 7th day, the number of PRL-cells was significantly increased ($p<0.001$).

Proliferative ratio of prolactin immunopositive cells

Double immunostaining with PCNA and PRL allowed the identification of proliferating PRL-cells (thick arrow in Figs. 2, 3).

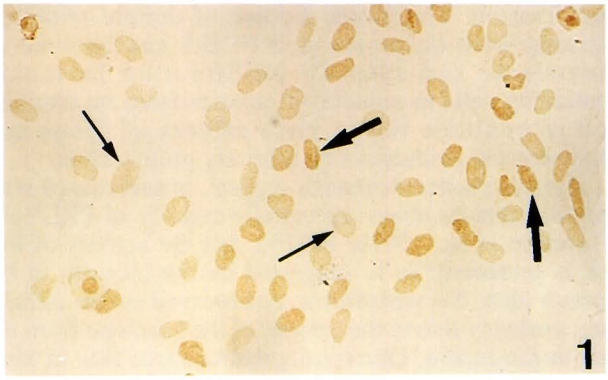
On the 3rd day of culture (see Table 1), the PRL-PCNA labelling index was 9.24±0.15%. At this time, 60.95±2.65% of PRL-cells and 8.68±0.12% of PCNA-positive cells were proliferating PRL-cells.

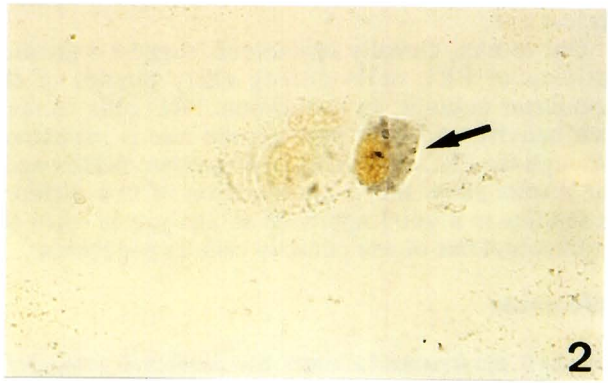
Significant differences of the proliferation rate were observed on the 7th day in relation to the 3rd day of culture; the PRL-PCNA labelling index and the percentage of proliferating PRL-cells in relation to the total PCNA cells were increased, but the percentage of proliferating PRL-cells in relation to the total PRL-cells was not modified (see Table 1).

Morphometric findings

On the 3rd day, the cellular area was 79.69±4.25 μm^2, and the nuclei occupied 36.86% of the total cellular surface (29.37±1.45 μm^2). According to their cellular area, two different cellular populations were observed, one with large cells ranging from 90.72 to 139.48 μm^2 and the other one with small cells ranging from 30.84 to 90.00 μm^2, that were the 56 and 44% of total PRL immunopositive cells, respectively. Differences in the reaction for PCNA were not found related to these populations.

On the 7th day, the cellular area was similar to that





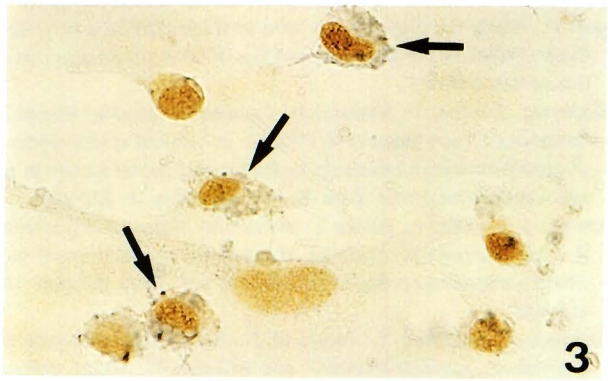


Table 1. Percentages of PCNA, PRL or PCNA and PRL cells in relation to the total cells per dish and percentages of PCNA and PRL cells in relation to the total PRL cells per dish (PRL&PCNA/PRL) or the total PCNA cells per dish (PRL&PCNA/PCNA), expresed as arithmetical mean±standard error of the mean.

	3rd day	7th day
PCNA cells	48.35±2.78	54.93±2.83
PRL cells	15.16±0.21	32.18±060*
PRL&PCNA cells	9.24±0.15	19.41±0.23*
PRL&PCNA/PRL cells	60.95±2.65	60.32±2.34
PRL&PCNA/PCNA cells	8.68±0.12	19.88±1.09*

*: $p<0.001$.

Table 2. Morphometric values (μm^2) observed in celullar, nuclear and cytoplasmic areas for PRL-cells on the different periods of incubation analyzed, expressed as arithmetical mean±standard error of the mean.

	3re day	7th day
Cellular area	79.69±4.25	76.52±2.03
Nuclear area	29.37±1.45	36.85±1.25*
Cytoplasmic area	50.32±2.83	39.85±1.25*
% of cell occupied by nuclei	36.86%	48.16%

*: $p<0.001$.

Fig. 1. Proliferating cell nuclear antigen (PCNA)-immunoreactive nuclei from a dish on the 7th day of incubation. Thick arrow: strongly-stained nucleus; thin arrows: weak nuclear immunoreaction. x 150

Fig. 2. Prolactin- and PCNA-stained cell from a dish on the 3rd day of culture (arrow). x 450

Fig. 3. Prolactin- and PCNA-stained cells from a dish on the 7th day of culture (arrows). x 450

observed on the 3rd (see Table 2), but the nuclei were increased in size and occupied 48.16% of the cell surface ($p<0.001$). Only one cellular population with cells ranging from 52.00 to 99.49 μm^2 was observed.

Discussion

PCNA is a protein associated with DNA polymerase-delta which is expressed in the G_1 phase, reaches a maximum in the mid-late stage of the S-phase and declines again during the G_2 and M phase of the cell cycle (Mathews et al., 1984; Tan et al., 1986; Prelich and Stillman, 1988).

This is also evident from the findings in pituitary monolayer cultures reported here, because 97.49% of the PCNA immunoreactive cells showed nuclear PCNA positivity and the different staining intensities observed were likely to be due to different concentrations of PCNA at different phases of the cell cycle. The occurrence of cytoplasmic PCNA reaction in 2.51% of the cells could be due to diffusion of PCNA from the nucleus into the cytoplasm during mitosis, according to the in vivo findings reported by Oishi et al. (1993), because the PCNA protein is known to have a 24-hour half-life in vitro system (Bravo et al., 1987) and it is detected in cells that have recently completed mitosis (Hall et al., 1990; Coltrera and Gown, 1991).

Various researchers reported in vitro cell kinetic analysis of pituitary cells: Komolov et al. (1988) analyzed the proportions of PRL cells in S-phase by means of ^{3}H-thymidine incorporation to DNA after treatment with TRH or somatostatin and found a direct inhibitory effect for somatostatin. An inhibitory effect for dopamine on proliferation of PRL cells has been reported (McComb et al., 1986).

Because in our study there are no hypothalamic inhibitory factors, the findings reported by the above-mentioned authors, could explain the increased proliferation rate of PRL cells during early culture phases found in the study reported here, which is maintained throughout the evolution of the culture and it was not dependent on the total PRL-cell amount in the dish.

In vivo, without analysis of the proliferative ratio, different percentages have been described for every pituitary cellular population (review in Dada et al., 1984), but no detailed study on monolayer cultures was performed. Our results suggest that at early periods of incubation the PRL cells were less abundant than in normal in vivo conditions. The high proliferation of these cells in vitro suggests an increase of this population after long incubation periods (this was confirmed in the present study) when the results on the 3rd day are compared to the results on the 7th day.

Only one work describing the percentage of classical cellular populations of the rat pituitary in proliferation exists in the literature. Oishi et al. (1993) analyzed, in vivo, the percentage of double immunostained cells for BrdU and pituitary hormones. According to these report, in male rats, PRL cells are 37.9% of total BrdU positive cells; when these data are compared with our percentages of PRL- and PCNA-positive cells, higher percentages of PRL- and BrdU-positive cells could be observed. Although these authors used PCNA as proliferative marker and reported it to be approximately a five-fold more sensitive technique than BrdU for detecting proliferating cells, they only estimated the percentages of prolactin proliferative cells by means of BrdU expression, not for PCNA expression. These differences in the methodology employed, in addition to the in vitro conditions reported in our study, could explain the differences observed. Our results are the first reported analysis of in vitro proliferation rate of PRL-cells using PCNA as marker.

However, our results, in a similar way that was described in vivo by Oishi et al. (1993), suggest that PCNA is a good marker for in vitro cellular pituitary proliferation. Therefore, the use of double immunostaining with hormones and PCNA can be used to investigate and identify proliferating hormone-containing cells in monolayer cultures from rat pituitary and to evaluate the in vitro effects of drugs and physiological modulators on pituitary proliferation.

The morphometric results suggest, in accordance with previous reports about the cellular activity of PRL cells (Carretero et al., 1991b, 1992b), that the activity of this cell is increased, as is suggested by the increase in the nuclear area. No variation were observed in the cellular size, probably due to the release of the hormone from the cell to the media. Our results demonstrate that in vitro cell proliferation of PRL-cells induces increase in nuclear size.

Our results, globally considered, suggest a growing activity of PRL cells during early phases of the monolayer cultures. In conclusion, PRL cells increase their activity, and their proliferation rate is maintained although the PRL-cells increase in number. Additionally, our results show that a combination of two different antibodies is a good approach to analyze in vitro the proliferation rate of one pituitary cellular population.

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