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Proliferative Activity of Pituitary Mammotrophs in Culture : a Morphological Study on DNA Synthesis Using In Vitro Bromodeoxyuridine Labeling Method†

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= Abstract = **The present study was designed to investigate the proliferative activity of mammotroph in the long-term monolayer cultures of male rat pituitaries by use of bromodeoxyuridine(BrdU)-labeling technique combined with double immunohistochemical staining. Rat anterior pituitary cells were exposed to 100 uM BrdU for 4 hrs at 4, 7, 12, 15, 20, 25 and 30 days in the primary cultures. After fixation with modified Carnoy's fixative, double immunohistochemical staining with anti-BrdU and anti-prolactin antibody was performed. It was shown that the ratio of BrdU-labeled mammotrophs per 100 mammotrophs(BrdU Labeling Index) was 8.2% at 4 day and 8.0% at 7 day in our cultures. Thereafter, it decreased until 30 days(1.7%). These results demonstrated that the increase in the proportion of mammotrophs observed in our previous monolayer cultures is caused, at least in part, by the cell division of mammortrophs.**

Key Words: *Mammotroph proliferation, Bromodeoxyuridine, Rat, Anterior pituitary cell culture, Double immunohistochemistry*

INTRODUCTION

The cell culture system is known to be a valid model for studying the dynamic properties of the hormone producing cells. Since the cellular reactions during the culture vary with the type of cells, it must first be investigated to understand the patterns of the cellular responses to the culture environment.

We had previously observed that the proportion of the mammotrophs increased while that of somatotrophs decreased during the monolayer pituitary cell cultures of the male rat pituitaries(Eoh *et al.* 1992; Lee *et al.* 1993). The results were consistent with the reports of other researchers such as Tixier-Vidal *et al.* (1975), Baker *et al.* (1976), and Shin *et al.* (1988). The possible hypotheses for these phenomena have been proposed by Olivier *et al.* (1974). First, multipotent stem cells remain in the adult anterior pituitary gland. Second, the fully differentiated cells may be able to undergo mitosis. Third, a fully differentiated cell can be converted to another type of fully differentiated cell.

Among these, the mitotic activity of mam-

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motrophs in culture was demonstrated by Lee *et al.* (1993) who used colcemid, the mitotic arresting drug. In addition, the proliferating activity of a cell population can be estimated by the thymidine index, which is expressed as the percentage of cells labeled by ^{3}H-thymidine (Baserga 1989). Since most pituitary cells which incorporate ^{3}H-thymidine into DNA subsequently divide, ^{3}H-thymidine labeling is an indirect estimate of cell mitosis. Although, the thymidine labeling index has been used as one of the most reliable criteria for the proliferating activity until now, it has several disadvantages including poor resolution and long exposure time. To overcome these problems, we used the bromodeoxyuridine(BrdU) labeling method in the present study. Bromodeoxyuridine, a synthetic thymidine analogue, incorporates into the replicating DNA, and visualization is possible by immunohistochemical staining using monoclonal antibody(Gratzner 1982).

The purpose of this study is to show further evidence of the mitotic activity of the mammotrophs in the monolayer pituitary cell cultures using BrdU-labeling.

Although there has been a report which used BrdU-labeling to study mammotroph proliferation during the postnatal period(Carbajo-Perez and Watanabe 1990), as far as we know, this is one of the first reports that applied the BrdU-labeling technique combined with double immunohistochemistry for the study of the mammotroph proliferation in the in vitro system.

MATERIALS AND METHODS

Animals

Sprague-Dawley male rats weighing 200-250 gm were housed in a temperature controlled (22-25℃) and artificially illuminated(lights on for 12 hrs) animal room. Food and water were available ad libitum. At least fifteen animals were killed between 09:00～12:00 h by cervical dislocation. The pituitary glands were removed aseptically. The neurointermediate lobe was discarded, and the remaining tissue was used in this study.

Cell dissociation and culture

The anterior lobe was cut into small fragments in sterile Hanks' balanced salt solution(HBSS, Gibco), transferred to a 15 ml conical centrifuge tube containing 5 ml HBSS, and allowed to settle out. The supernatant was decanted and replaced with 10 ml HBSS containing 0.2 % collagenase(CLS, Worthington Biochemical Co.) and 50 μg/ml deoxyribonuclease(Type IV, Sigma) and incubated for 1 hr at 37℃ in a water bath. The cells were harvested by centrifugation for 10 min at 100 g. After two more washes in HBSS, the cell suspension was passed through a stainless steel mesh(pore size = 50 μm). The cells were finally suspended in a Dulbecco's Modified Eagle's Medium(DMEM, Gibco) containing 10 % fetal calf serum(Gibco), 2 mM L-glutamine, 25 mM HEPES, and 40 μg/ml gentamycin.

Viable cells were counted by 0.2 % trypan blue and adjusted to a concentration of 3×10^5 cells/ml. 100 μl cell suspension was plated onto polylysine(0.1 mg/ml), coated fluorocarbon coverslips(Allied Chemical), placed in plastic culture petri dishes. The cells were incubated in a humidified atmosphere of 5 % CO_2- 95 % air at 37℃. After the initial 48-72 hr incubation which allowed the cells to adhere to the coverslips, fresh culture medium was added to overflow the petri dishes. Thereafter, media were changed every 3 days and the cell culture was maintained up to 30 days.

BrdU-labeling and detection

BrdU(Sigma) was added to culture medium to adjust the concentration to 100 μ M at 4, 7, 12, 15, 20, 25 or 30 days in culture. After 4 hr incubation with BrdU, the coverslips were washed with phosphate buffered saline(PBS), and fixed in a modified Carnoy's fixative(methanol:acetic acid=3:1 V/V) for 30 min at RT. Cells were washed with PBS, treated with 1 N HCl for 10 min and with 2 N HCl for 15 min for DNA denaturation. 0.1 M borax solution was used for neutralization followed by inactivation of endogenous peroxidase by immersing the coverslips in methanol containing 0.3% H_2O_2 for 30

min. Cells were incubated in 1:100 normal horse serum diluted in PBS for 30 min at RT. BrdU incorporation was detected by incubating the cell with 1:50 anti-BrdU monoclonal antobody for 1 hr, biotinylated anti-mouse gamma globulin for 1 hr, ABC reagent(Vector, ABC kit PK6102) for 1 hr and 3,3'-diaminobenzidine(DAB)(Sigma) for 1 min.

Immunohistochemical staining for prolactin

In order to visualize mammotrophs in culture, the pituitary cells which were already stained with anti-BrdU antibody were washed with PBS, incubated with 1:5000 rabbit anti-rat prolactin antibody(UCB) at 4℃ overnight, biotinylated anti-rabbit antibody for 1 hr, ABC complex(Vectastain kit) pk6101 for 1 hr and benzidine dihydrochloride (BDHC, Levey *et al.* 1986) for 3 min. BDHC were used to produce blue-colored products.

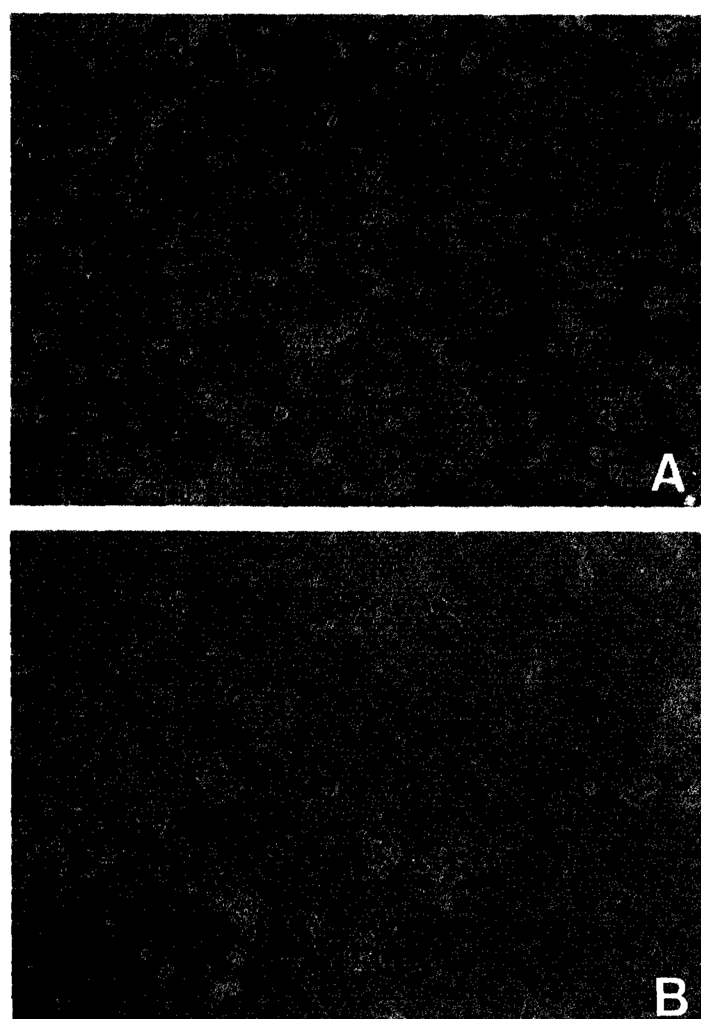

Fig. 1. Inverted photomicrographs of pituitary cells in the primary culture. Note two types of cells based on nuclear morphology. The pituitary secretory cells with smaller nuclei are more refringent, dense and round, while the cells with larger nuclei are the typical squamous fibroblasts. ×100. A : 5 days in culture, B : 20 days in culture

Quantitation of BrdU labeled mammotrophs

The numbers of double immunostained mammotrophs were counted under a light microscope at a magnification of 400×. The bromodeoxyuridine labeling index(BLI = ratio of the number of BrdU-labeled mammotrophs per 100 mammotrophs) was calculated and analyzed.

Fig. 2. Cultured mammotrophs after double immunocytochemical staining with anti-BrdU(brown) and anti-prolactin(blue) antibodies. ×400. A : BrdU-labeled and unlabeled mammotrophs, B : BrdU-labeled binucleated mammotrophs

RESULTS

Immunohistochemical staining results for BrdU and prolactin

Anterior pituitary cells were maintained in a monolayer culture system for up to 30 days. The types of cells could not be distinguished by shapes(Fig. 1). With the double immunohistochemical staining, cytoplasmic prolactin granules were identified by the presence of the blue-colored BDHC reaction products and the BrdU-incorporation in the nuclei by the presence of the brown-colored DAB reaction products. Anti-BrdU immunoreactivity was found in the nuclei of other cell types as well as mammotrophs(Fig. 2, 3) The patterns of nuclear BrdU staining in mammotrophs were various. That is, it showed a diffuse but unevenly stained appearance in the overall nucleoplasm(Fig. 4A), preferential localization close to the nuclear boundary and the nucleolus(Fig. 4B), or limited localization close to the nuclear membrane(Fig. 4C).

Changes of the BLI of mammotrophs during the culture period

Table 1 shows the time course of BLI of mammotrophs cultured by this method. It showed that the mitotic activity could be found from 4 to 30 days in culture. Mammotrophs showed BrdU-labeling in 8.2 percent at 4 days

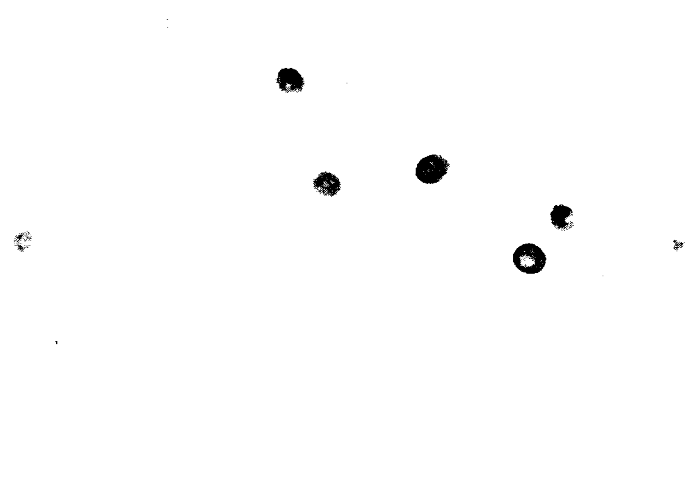

Fig. 3. Photomicrographs of BrdU incorporated anterior pituitary cells. Immunoperoxidase stain for BrdU followed by counterstain with hematoxylin shows many labeled nuclei. ×200.

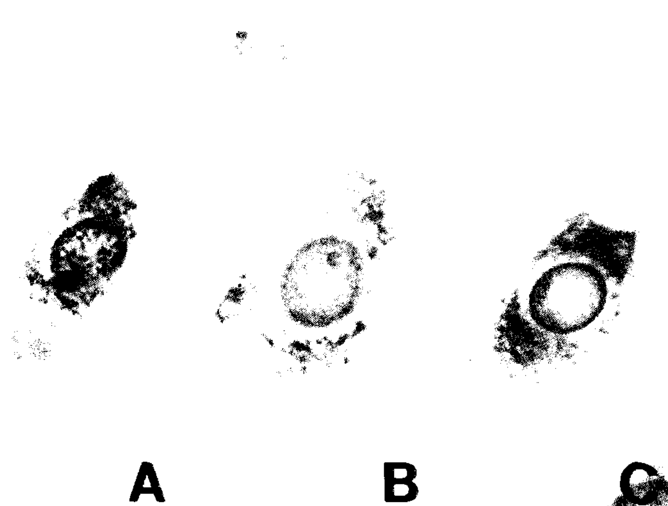

Fig. 4. Nuclear BrdU staining patterns of cultured mammotrophs. ×1,000.
A : The staining is shown throughout the nucleus. Heterochromatin is more heavily labeled than interchromatin.
B : The labeling is localized preferentialy close to the nuclear boundary and around the nucleolus.
C : Localization close to the nuclear membrane is observed.

Table 1. The BrdU labeling index of mammotrophs during the long-term monolayer pituitary cell cultures.

Days in culture	BrdU labeling index#
4	8.2 ± 1.0
7	8.0 ± 3.0
12	4.6 ± 1.7*
15	4.4 ± 2.0
20	3.0 ± 0.9
25	3.0 ± 0.9
30	1.7 ± 0.3

Percent of BrdU labeled mammotrophs per 100 mammotrophs. These values are the 95 % confidence intervals. (mean ± t0.025×SE)

* The value is significantly different from those of the earlier days in culture($p<0.05$).

in culture and in 8.0% at 7 days.

However, at 12 days the value of BLI was reduced significantly ($P < 0.05$). Thereafter, the values of the BLI decreased until it showed 1.7% at 30 days.

DISCUSSION

Compared to other morphological methods which have been used for the study of pituitary cell proliferation, BrdU-labeling seems to be the most suitable technique because of its simplicity, reproducibility and high resolution. Moreover, it allows the detection of specific cell types when combined with double immunocytochemical staining. However, there has been no BrdU-labeling study, to our knowledge, that was able to distinguish the specific type of BrdU-labeled pituitary cells in the culture system.

In the present study, we were able to demonstrate the proliferative activity of mammotrophs in the monolayer cultures of male rat pituitaries by use of BrdU-labeling followed by double immunohistochemical staining. Optimization of protocol was performed as follows:

The commonly used concentration of BrdU has usually been 10 μM, but more than 10 μM of BrdU has not been reported to cause problems (Beisker *et al.* 1987). After several preliminary studies, we determined the optimal final concentration of BrdU as 100 μM. The minimum incubation time with BrdU was reported to be 6 minutes in case of plasmacytoma cells in vitro (Gratzner 1982). However, in the present study, at least 1.5 hours was required for the detection of BrdU incorporation with monoclonal antibody. Since the obtained BLI was lower than 7 percent, which was not enough to evaluate the differences in the BLI during the culture period, we increased the incubation period up to 4 hrs.

For fixation, cold methanol has been found to be better than 70% ethanol when used for NACM6 and PRE-B cell lines to show good morphological preservation and successful double immunohistochemical stain for BrdU and other antigens(Compana at al. 1988). The experiment by McNicol *et al.* (1990), in which 70% ethanol was used as a fixative, could not successfully show the results of the double immunohistochemical stain. Therefore, we used the mixture of methanol: acetic acid(3:1) which was used by Gratzner(1982).

The present study confirmed that mammotrophs are capable of mitosis and showed the changes of mitotic activity during the long-term pituitary cell cultures. From 4 to 7 days in culture, BLI was as high as 8.2% and then decreased. The time course of BLI was similar to that of our previous study using colcemid (Lee *et al.* 1993), but the magnitude was different. It was conceivable that the mitotic rate was higher with colcemid, because the longer incubation time was used with colcemid(24 hrs) instead of 4 hrs for BrdU labeling. Also, the DNA-replication and mitotic rates at a given point of time cannot be expected to be exactly same. However, our studies did not reveal the relative contribution of mammotroph mitosis and conversion of sommatotrophs into mammotrophs to the increase in the proportion of mammotrophs in the culture system.

It is known that the prolactin is secreted in high levels in the initial period(4 days) of the primary pituitary cultures due to the removal of hypothalamic inhibition(Linda *et al.* 1979). Although the underlying mechanism has still not been fully elucidated, it was suggested that the increased secretion of prolactin is attributed to the increased number of mammotrophs (Daniel *et al.* 1964; Antunes *et al.* 1980) as well as to the increased prolactin synthesis from each cell. Therefore, our results raised a question as to whether or not the high mitotic activity of mammotrophs demonstrated in the early period of our studies accompanied the disconnection from the hypothalamus. However, we cannot exclude the possibility of mitotic effect of the steroids in the fetal bovine serum used for our culture media. Further studies are required to elucidate the mechanism of in vitro mammotroph proliferation.

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