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New insights into the cytodynamics of the hamster Harderian gland as provided by the bromodeoxyuridine-labelling method

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Summary. The fourth week of postnatal life is a critical point in the development of the hamster Harderian gland. During this week, cells with large lipid vacuoles (type-II cells) appear in the male gland, marking a morphological sex difference that is notorius in adult animals. The origin and fate of type-II cells are controversial. To gain insight into the mechanisms by which type-II cells become a major cell type in the gland of adult male hamsters, bromodeoxyuridine (BrdU) labelling was used to assess the proliferative activity of both types of glandular cells in 28-day-old animals. To search for possible sex differences in the proliferative activity of this gland, female animals of the same age as the males were also studied. No difference was found in the overall labelling index (BrdU-labelled cells/100 cells) between males (1.8±0.1%) and females (1.5±0.1%). In the gland of the males, the specific labelling index of type-II cells (3.4±0.4%) was significantly higher than that of type-I cells (0.9±0.2%). Interestingly, the proportion of type-II cells present in the male glands at this age (36.6%) was significantly lower than that of type-I cells. Our results strongly suggest that the proliferation of type-II cells, rather than a continuous differentiation of these cells from preexisting type-I cells, is a major event in the achievement of the mature form of this gland. The results reported here counsel a reappraisal of current theories about the cytodynamics of the hamster Harderian gland.

Key words: Cell proliferation, Harderian gland, Development, Hamster

Introduction

The Harderian gland is an orbital gland which is particularly well developed in rodents. Marked sex differences regarding structure and possibly function are found in the gland of adult Syrian hamsters, although the glands of males and females are strikingly similar shortly after birth (Review by Payne, 1994). From a morphological point of view, two types of secretory cells (type-I and type-II cells) are present in the gland of adult male Syrian hamsters, while only one cell type (type I-like cells) is present in the mature female gland (Hoffman, 1971; López et al., 1993). The appearance of type-II cells during the fourth week of postnatal life heralds the shift towards the male phenotype (López et al., 1992). On the 20th postnatal day, type-II cells are almost undetectable, but by the end of the first month these cells account for more than one third of the total number of secretory cells of the gland. The question as to whether these cell types are different forms of the same cell or whether they are independent of one another remains controversial (Payne, 1994).

This study was carried out in an attempt to ascertain the mechanisms by which type-II cells become a major cell type in the gland of adult males. With this in mind, 28-day-old male hamsters- in which the mature form of the gland has not yet been achieved, but in which a significant proportion of type-II cells is already present- were selected, and the proliferative activity of the different cell types of the Harderian gland was assessed after *in vivo* labelling with bromodeoxyuridine (BrdU). To explore the existence of possible sex differences in the overall proliferative activity of the gland, samples from females of the same age as the males were also studied.

Materials and methods

Animals

Eight 28-day-old Syrian hamsters (*Mesocricetus auratus*), four males and four females, were used. After weaning, animals were caged according to sex under conditions of controlled temperature (23±2 ºC) and lighting (lights on from 06.00 to 20.00 h). Food

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and water were freely available. On the 28th post-natal day (12.00 h) animals received an intraperitoneal injection of 100 mg/kg body weight of BrdU (Sigma, St. Louis, MO, USA) in saline (10 mg/ml) and were killed an hour later by decapitation under ether anesthesia.

Tissue preparation and immunohistochemistry for bromodeoxyuridine

Harderian glands were dissected out, fixed in Carnoy's solution for 30 minutes, dehydrated and embedded in Paraplast. Four to five µm-thick sections were obtained. Sections were dewaxed, thoroughly washed in water, and BrdU-labelled nuclei revealed as described elsewhere (Carbajo et al., 1992). DNA was denatured by incubating in 95% formamide in 2X standard sodium citrate at 60 ºC for 45 minutes. Samples were then incubated overnight with monoclonal antibody to BrdU (1:20; Dakopatts, Glostrup, Denmark) in a most chamber at 4 ºC, followed by incubation with biotinylated anti-mouse IgG (1:100, 60 min, room temperature; BIOMEDA) and streptavidin-peroxidase complex (45 minutes, room temperature; BIOMEDA). The final reaction product was revealed with 3,3'-diaminobenzidine (DAB, Sigma) (10 mg DAB in 50 ml of 0.05M Tris-hydrochloric buffer plus 50 µl of 0.3% hydrogen peroxide). Preparations were lightly counter-stained with Gill's haematoxylin and mounted with DPX. Sections from animals which were not exposed to BrdU served as negative controls.

Data processing

For morphometric purposes a light microscope connected to an MIP image analyzer (MICROM España, Spain) was used. BrdU-labelled and unlabelled type-I and type-II cells (males) and total number of labelled cells (females) were quantified using a x40 objective lens. The different types of labelled and unlabelled cells were identified on the screen and recorded using a digital tablet. Fifty microscope fields from each animal were studied (about $3x10^3$ cells per animal). The overall labelling index of the gland (LI= number of BrdU-labelled cells per hundred secretory cells) was calculated both in males and females. Additionally, the specific LI for each cell type (number of labelled cells from each secretory cell type per hundred cells of the same cell type) and the contribution of either type of secretory cell to the total proliferative activity of the gland (proportion of labelled type-I and type-II cells to total number of BrdU-labelled secretory cells) was calculated in males. Cell density (total number of cells/mm^2 of tissue section) and labelling density (number of labelled cells/mm^2 of tissue section) were also calculated.

Student's t-test for paired and unpaired data was used as appropriate for two-group comparisons. Proportions were compared after the inverse sine transformation.

Table 1. Morphometric data obtained from sections of the Harderian gland of 28-day-old male and female hamsters. Values are mean±SEM (n=4 each group).

	FEMALES	MALES		
	Overall	Overall	Type I	Type II
%	100	100	63.4±1.2	36.6±1.2
LI	1.5±0.1	1.8±0.1	0.9±02	3.4±0.4
CD	$3.1x10^3$±129	$2.8x10^3$±70	$1.8x10^3$±73	$1.0x10^3$±23
LD	45.1±3.0	50.1±3.1	15.6±3.3	34.6±3.4

LI: labelling index; CD: cell density; LD: labelling density.

Results

The immunocytochemical reaction with BrdU antibody permitted an easy identification of labelled nuclei. As a whole, BrdU labelling was similar in male and female glands (Fig. 1a,c). Either male type-I or type-II cells (Fig. 1b) and female glandular cells appeared scattered throughout the gland (Fig. 1d).

Morphometric data are shown in Table 1. The morphometric study showed that the overall BrdU-LI was slightly higher in males (LI=1.8±0.1%) than in females (1.5±0.1%) although this difference was not statistically significant (p=0.09). No sex difference was found regarding cellular density or labelling density. In the male gland the LI of type-II cells (3.4±0.4%) was significantly higher ($p<0.05$) than that of type-I cells (0.9±0.2%). The contribution of type-II cells to the overall proliferative activity of the gland (69.2±6.4%) was greater than the contribution of type-I cells (30.8±6.4%), even though the type-I cell population (63.4±1.2%) clearly outnumbered that of type-II cells at this age (Fig. 2).

Discussion

It is generally accepted that the Harderian gland of the male hamster is made up of two types of glandular cells which differ in morphology and possibly in function, while in females only one type of glandular cell is present (Payne, 1994). There is clear hormonal control of these cell types. Experimental evidence strongly supports that type-II cells are under androgenic control: 1) type-II cells disappear after orchidectomy (McMasters and Hoffman, 1977; Payne et al., 1977), this effect being prevented by androgen replacement (Payne et al., 1977); 2) androgen administration to female hamsters results in the appearance of type-II cells; and 3) the appearance of type-II cells during the fourth week of postnatal life is paralleled by increasing serum testosterone concentrations during this period (Rodríguez-Colunga et al., 1993).

Although it is generally agreed that type-II cells are subject to androgenic control, the origin and eventual fate of these cells is controversial. Early theories (Hoffman, 1971) suggested that type-II cells would be degenerating cells that could be secreted in toto into the lumen. Holocrine secretion of type-II cells has also been

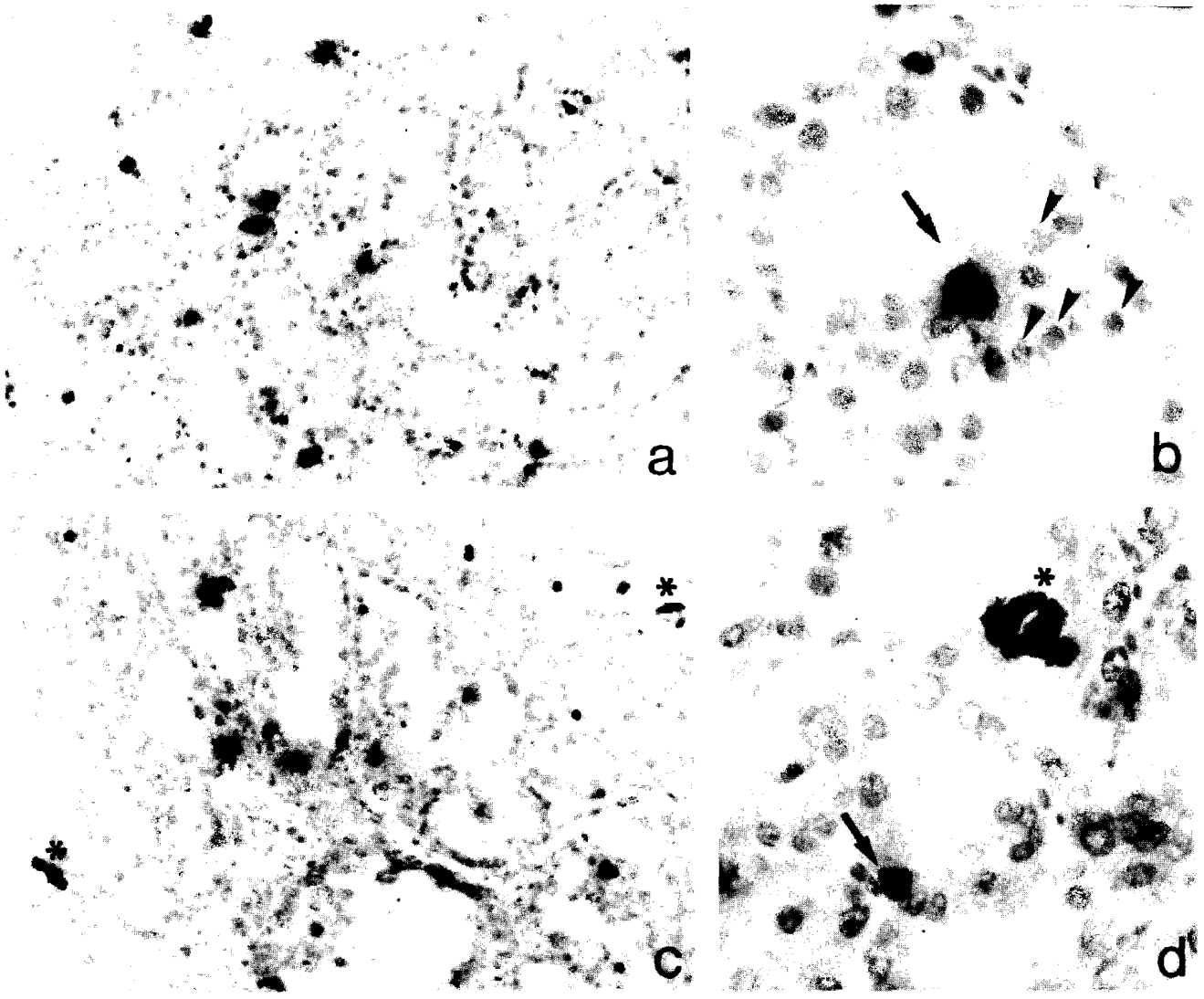

Fig. 1. Parts of sections of the Harderian gland from 28-day-old male (**a,b**) and female hamsters (**c,d**). BrdU-labelled cells are easily identifiable, even at low magnification (a,c). Some rough deposits of porphyrins are seen in the glands of females (asterisks). In the gland of males, type-II cells (highly vacuolated cells, arrowheads) are easily distinguishable from type-I cells at a higher magnification. Note the presence of a type-II cell (b) and a female cell (d) labelled with BrdU (arrows). a, c, x 130; b, d, x 530

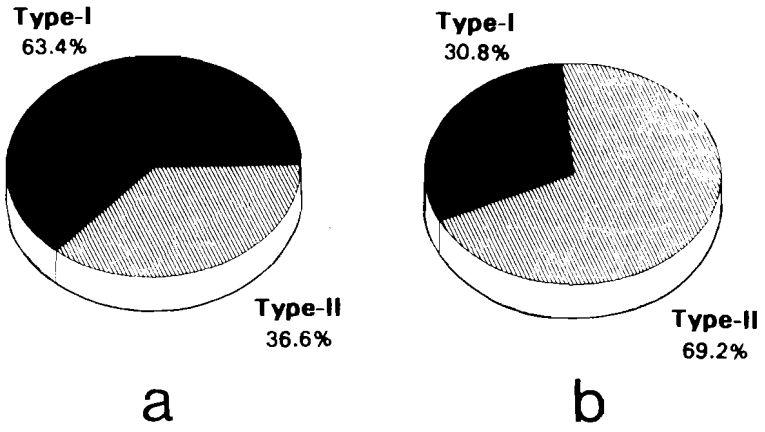

Fig. 2. The proportions of type-I and type-II cells in he Harderian gland of 28-day-old male hamsters (**a**) as well as the proportion of the total number of cells labelled with BrdU belonging to each cell type (**b**) are shown. Note that while the proportion of type-I cells is higher than that of type-II cells, the contribution of the latter to the overall LI of the gland is greater.

considered as a possible mechanism by which type-II cells disappear after castration of golden hamsters (Rodríguez-Colunga et al., 1993). It has also been proposed that type-I and type-II cells would correspond to different activity levels of the same cell type (Payne et al., 1992). In this sense, a cell cycle activity with morphological correlates would exist within the Harderian gland (Payne, 1994). This notion has been strengthened by the fact that no separate precursors for the different cell types have been identified in studies on the postnatal development of this gland (Bucana and Nadakavukaren, 1973; López et al., 1992, 1995).

Despite all the available information, some important questions remains to be elucidated: (i) Is there is a continuous process of differentiation from type-I to type-II cells to maintain the population of type-II cells or, by contrast, once type-II cells differentiate, are they a self maintained population? (ii) Do transdifferentiation phenomena exist through which - according to functional demands - type-I and type-II cells are interconverted, as can happen with well differentiated cells of the anterior pituitary gland (Horvath et al., 1990)? It seems reasonable to assume that detailed analysis of the proliferative activity of the hamster Harderian gland would shed further light on these issues.

The fourth week of postnatal life is of particular relevance in the development of the Harderian gland of male hamsters. On the 20th postnatal day, type-II cells are almost undetectable, but by the 30th day these cells account for more than one third of all the secretory cells of the male glands. Twenty-eight-day-old male and female hamsters were chosen for our study because although the mature form of the gland has not yet been achieved at this age, a significant proportion of type-II cells is already present in the male gland, and the proliferative activity of the gland is presumably still high.

Few and contradictory data are available on the cellular proliferation of the hamster Harderian gland. Most data available are based on mitosis counts. In some reports (Bucana and Nadakavukaren, 1972) no mitotic figures are found beyond the third week of postnatal life while in others (López et al., 1992) the number of mitosis is still high during that week. Counting of mitotic figures in semithin sections (Rodríguez-Colunga et al., 1993) and methacrylate sections (López et al., 1996) from animals of similar age (30- and 27-day-old animals respectively) gave quite different results (0.87±0.1 and 0.98±0.1 mitoses/mm^2 in male and female glands in semithin sections, and 6.0±0.6 and 5.7±0.6 mitosis/mm^2 in methacrylate sections). Different processing of the samples and different criteria for the selection of mitotic figures may be at the bottom of the above mentioned differences.

To evaluate the specific proliferative activity of different cell types in a tissue in which the overall proliferative activity is not particularly high, as is the case of the Harderian gland at this age, it is of great importance to have a method permitting the identification of the greatest possible number of proliferating cells. Good morphological preservation would also be required in order to identify the proliferative activity of the different morphological cell types. Taking into account the different length of the various phases of the cell cycle, evaluation of the number of S-phase cells would afford higher absolute numbers than those obtained by counting mitoses. As already reported by Messier and Leblond (1960), the proportion of S-phase cells labelled with (^{3}H)-thymidine is three- to eight-times greater than the proportion of mitotic cells.

The BrdU-labelling of S-phase nuclei and the immunocytochemical detection of incorporated BrdU is a simple and reliable method for evaluating cellular proliferation (Silvestrini et al., 1988; Böswald et al., 1990). This method has become a useful alternative to classic techniques such as mitotic counting and the thymidine labelling index because of the difficult quantification of the former and complexity of the latter. In recent years BrdU-labelling has been extensively used for the study of cellular proliferation under different experimental conditions and has proved to be a technique of choice to study the proliferative activity in growing tissues (Carbajo-Pérez and Watanabe, 1990; Ikeda and Yoshimoto, 1991; Carbajo et al., 1992).

Direct comparison cannot be made between our data and previously reported data on cellular proliferation in the Harderian gland because different experimental methods have been used. The number of identifiable proliferating cells is higher in our study than in any previous reports; the number of BrdU-labelled cells ranged from 45 to 50 cells/mm^2 while in the best of cases mitotic counts gave no more than five to six mitoses/mm^2 (López et al., 1995). The relatively high number of BrdU-labelled cells we found in the Harderian gland permitted the evaluation of the proliferative activity of the two cell types of males with reasonable quality assurance.

The overall LI found here for the Harderian gland of 28-day-old male and female hamsters suggests that these glandular cells belong to an expanding cell population, as has been reported for most glandular tissues (Messier and Leblond, 1960). If this were true of the glandular cells in females and of the type-I cells in males, when analyzed separately, the LI of type-II cells would better fit that of a renewal cell system. Although this is a tempting suggestion, the high LI found here for type-II cells might also correspond to a burst of proliferative activity of type-II cells at this critical point of development.

It can be concluded that rather than a continuous differentiation from preexisting type I-cells, proliferation of type-II cells is the major factor leading to the mature form of the hamster Harderian gland. However, it cannot be ruled out that differentiation from type-I cells is the first step towards the type-II cell. Similarly, trans-differentiation phenomena should not be overlooked as an alternative mechanism through which the gland might adapt to functional demands or to changes in the androgenic environment. In the light of the findings

reported here a reevaluation of the existing theories on the cytodinamics of the Harderian gland certainly seems to be warranted. Longitudinal studies ranging from birth to adulthood together with studies on proliferative activity after modifications in the androgenic environment are currently in progress with a view to gaining a deeper insight into the cytodynamics of the hamster Harderian gland.

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