Structural and Ultrastructural Immunocytochemical Study of Somatomammotrophs in Ovine Adenohypophysis: Age and Lactation Influences

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Summary. The influences of age and lactational period on the distribution, number and structure of somatomammotrophs (SMTs) and the relationships to changes in somatotroph and lactotroph populations in ovine were studied using immunocytochemical procedures with light and electron microscopy as well as morphometric techniques. Adenohypophyseal glands of 15 individuals of the Segureña breed (female lambs and ewes in anoestrus or at different stages of milk production) were used. SMTs were always seen in the adenohypophysis of sheep, but were scarce in number and more frequently distributed in the anterior area of the gland. Their number decreased with age, increased at the beginning of the lactation, and decreased again in late lactation. Ultrastructurally these cells showed variable morphology and stored GH and PRL in different secretory granules. Data presented in this report suggest that SMTs are a stage between PRL and GH producing cells.

Growth hormone and prolactin were once thought to be produced and secreted by two different cell types. The application of immunocytochemical techniques to light microscopy (TASHJIAN et al., 1970; ITO et al., 1972) and electron microscopy (PAPKA et al., 1986; NIKITOVITCH-WINER et al., 1987; LOSINSKI et al., 1989; THORPE et al., 1990; SÁNCHEZ et al., 1994) has allowed the identification of a cell type which produces both hormones, the SMT cell. These cells have been studied in sheep (THORPE et al., 1990), goats (SÁNCHEZ et al., 1994) and cattle (FUMAGALLI and ZANINI, 1985; HASHIMOTO et al., 1987). Their number varies according to the hormonal and the reproductive state of the animal. In rats, ISHIBASHI and SHINO (1989) found them only during gestation, while PORTER et al. (1990) reported a decrease at that stage and an increase at the beginning of the lactation. STRATMANN

et al. (1974) described their presence in rats treated with oestrogens, and PASOLLI et al. (1994) noted their rare occurrence in ovariectomized, orchidectomized and whole rats. In contrast, KINEMAN et al. (1991) observed a considerable (21%) rise in castrated calves treated with GRF and TRH. Independently of the hormonal state, the presence of this cell type has not been corroborated in rats (KUROSUMI et al., 1986; SMETS et al., 1987), ovine fetuses (STOKES and BODA, 1968) or lambs (ORTMAN, 1987). While its functional significance has yet to be fully established, PORTER et al. (1990, 1991) claim that it is a bidirectional, transitional cell type occurring in the conversion of GH cells to PRL cells, or *vice versa*.

The aim of this study is to determine the influence of the age and lactation on the distribution, number, structural and ultrastructural characteristics, and hormone storage of SMTs and their relationships to changes between PRL and GH cell populations, applying immunocytochemical techniques for light and electron microscopy.

MATERIAL AND METHODS

The adenohypophysis of 15 animals of Segureña ovine breed were studied: 3 female lambs of 24 kg and aged 3 months (prepuber), 3 ewes in anoestrus of one year, 6 ewes in lactation—3 animals at 7 days lactation (early lactation), the other three in the third month of lactation (late lactation), and 3 ewes in the third month of lactation and 7 days after weaning.

Animals were anesthetized with Pentotal and the head was perfused through both carotids to direct systolic pressure during 15–20 min. First it was introduced into a washing solution for 1 min (0.8%

of sodium chloride + 0.4% of dextrose + 0.8% of sucrose + 1% of sodium nitrite in PBS 0.12 M, pH 7.4) and subsequently fixed in 4% paraformaldehyde and 0.5% glutaraldehyde in PBS 0.12 M, pH 7.4. The gland was sectioned in two halves following the sagittal plane, one being used for structural study and the other for ultrastructural study. Samples were obtained during April and July.

Light microscopy

The sagittal half of each gland was washed in cacodylate buffer (0.2 M, pH 7.2 with 1% of sucrose) for 1 h, refixed in sublimate formalin (Gerad's liquid), and embedded in poliwx (Difco). Sixty sagittal serial sections (4–5 μ m thick) were mounted on slides. Double immunolabeling was done combining the indirect immunoalkaline phosphatase technique (AP) and ABC immunostaining technique (AP-ABC). In the first immunoreaction after treatment of the samples with normal goat serum (Sigma) at a 1/30 dilution, the samples were incubated with rabbit anti-ovine GH (1:1000, UCB) for 60 min at 32°C. Biotinylated swine anti rabbit IgG (DAKO) was used as the second antibody at a 1/250 dilution in TBS (0.05 M, pH 7.6) for 20 min. The slides were rinsed and incubated for 30 min with the anti-biotin alkaline phosphatase complex (Sigma) diluted 1:100 in the same buffer for 30 min. After rinsing with TBS, alkaline phosphatase

was developed with naphthol-AS-MX-phosphate (0.1 M Tris buffer pH 8.2 containing 0.24 mg levamisole-Sigma- and 1 mg fast blue salt -Sigma). Then sections were treated for 1 h at 60°C with paraformaldehyde vapour after the first immunoreaction. Second immunoreaction samples were incubated with rabbit anti-ovine PRL (1:1000, UCB) for 60 min at 32°C. Swine anti-rabbit IgG (Sigma) was used as the second antibody at a 1:250 dilution. The reaction was then continued with PAP soluble complex (Sigma) at a dilution of 1:100. The reaction was visualized with 3-3 diaminobenzidine tetrahydrochloride (DAB, Sigma). Slides were not counterstained, and were mounted with glycer gel (Dako).

Electron microscopy

The remaining half of each gland was divided into three portions: anterior, medium and posterior. Samples were taken from the central and peripheral regions of each portion, and then fixed by immersion in 3% glutaraldehyde in PBS, pH 7.4 and postfixed in 1% osmic tetroxide for 2 h at 0°C. The tissue was then dehydrated in graded alcohol and propylene oxide, and embedded in Epon.

For the immunocolloidal gold complex method (ROTH, 1983), ultrathin sections were etched for 30 min with sodium metaperiodate (BENDAYAN and ZOLLINGER, 1983). After washing with TBS, sections were succes-

Fig. 1. Proportions of pituitary cells from prepuberal (P), anoestrus (A), early lactating (EL), late lactating (LL), and after weaning (AW) female ovine which released GH, PRL and SMT.

sively incubated at 32°C in the following solutions diluted in TBS: normal goat serum, 1:30 for 20 min (Sigma); rabbit anti-ovine GH, 1:1000 for 60 min, goat anti rabbit immunoglobulin coupled to Au 10 nm (1:40, Serva, Germany) for 30 min. Ultrathin sections were treated for 1 h at 60°C with paraformaldehyde vapour after the first immunogold stain according to a slightly modified method by WANG and LARSSON (1985). The second immunogold reaction was completed using the alternative first antibody (anti-ovine PRL, 1:5000) and a different size of colloidal gold particle (20 nm). Grids were routinely contrasted with uranyl acetate and lead citrate.

For both light and electron immunocytochemistry the specificity of the staining was checked as follows: 1) incubation of normal rabbit serum instead of

specific antiserum; 2) adsorption of the specific antiserum with its corresponding homologous hormone. No immunostaining was recognized these controls.

Morphometry

The numerical densities of GH, PRL and SMT cells were calculated in five fields of 10,000 μm^2 per section chosen randomly from ten sections of pars distalis, separated from each other by 50 μm . A total of 50 fields per animal were evaluated.

The other parameters (area and cellular diameter—in light microscopy, numerical density of the secretory granule and granular diameter—in electron microscopy) were calculated with an Image Analyzer Computer (IMCO 10 Kontron Bildanalyse) utilizing

Table 1. Mean $\pm$ standard deviation of different parameters of GH cells in ovine Segureña breed. Data of prepuberal (females), ewes in anoestrus and lactation (early lactation, EL; late lactation, LL; and after weaning, AW).

	Prepuberal	Anoestrus	EL	LL	AW
CD	11.59 $\pm$ 2.08*	13.52 $\pm$ 2.44**	11.71 $\pm$ 2.24	11.37 $\pm$ 2.36	11.71 $\pm$ 3.23
CA	60.98 $\pm$ 16.67*	90.10 $\pm$ 23.91**	61.43 $\pm$ 17.02***	60.40 $\pm$ 14.16***	56.43 $\pm$ 20.07
NvC	27.70 $\pm$ 7.96*	20.74 $\pm$ 9.96	18.84 $\pm$ 5.86	21.46 $\pm$ 7.75	22.00 $\pm$ 6.92
GD	368.4 $\pm$ 81.23*	421.5 $\pm$ 84.50**	380 $\pm$ 85.30	378 $\pm$ 78.98	363 $\pm$ 82.42
VvG	15.48 $\pm$ 6.32	15.29 $\pm$ 5.57	12.38 $\pm$ 6.22**	16 $\pm$ 7.89	9.86 $\pm$ 5.41**
NvG	2.38 $\pm$ 0.80*	1.70 $\pm$ 0.58	2.27 $\pm$ 0.88	1.73 $\pm$ 0.45***	1.89 $\pm$ 0.48***

*Significant differences between prepuber and anoestrus ewes groups, ** Significant differences between anoestrus and lactating or lactating and anoestrus groups, *** significant differences between lactating groups. (* $P < 0.01$). Parameters: cellular diameter in μm (CD), cellular area in μm^2 (CA), numerical density of GH cells in 10000 μm^2 (NvC), granular diameter in nm (GD), volume density of secretory granules in μm^3 (VvG), numerical density of secretory granules in μm^3 (NvG)

Table 2. Mean $\pm$ standard deviation of different parameters of PRL cells in ovine Segureña breed. Data of prepuberal (females), ewes in anoestrus and lactation (early lactation, EL; late lactation, LL; and after weaning, AW).

	Prepuberal	Anoestrus	EL	LL	AW
CD	13.11 $\pm$ 2.77*	14.12 $\pm$ 2.97	12.36 $\pm$ 2.42**	12.89 $\pm$ 2.30**	13.96 $\pm$ 3.32***
CA	68.82 $\pm$ 20.84*	89.30 $\pm$ 25.43**	66.09 $\pm$ 15.47***	75.97 $\pm$ 18.85***	84 $\pm$ 28.88***
NvC	38.94 $\pm$ 7.77*	48.18 $\pm$ 9.95	54.38 $\pm$ 7.33**	53.10 $\pm$ 11.59**	47.70 $\pm$ 10.84***
GD	375 $\pm$ 88.86*	295 $\pm$ 93.21**	442 $\pm$ 101.20	403 $\pm$ 103.20***	349 $\pm$ 78.32***
VvG	9.05 $\pm$ 5.48*	4.54 $\pm$ 3.32**	13.88 $\pm$ 5.96	14.55 $\pm$ 5.58***	8.68 $\pm$ 8.19
NvG	1.63 $\pm$ 0.49	1.94 $\pm$ 0.74**	1.41 $\pm$ 0.49	1.02 $\pm$ 0.50***	1.5 $\pm$ 0.77

*Significant differences between prepuberal and anoestrus ewes groups, **Significant differences between anoestrus and lactating or lactating and anoestrus groups, ***significant differences between lactating groups. (* $P < 0.01$). Parameters: cellular diameter in μm (CD), cellular area in μm^2 (CA), numerical density of GH cells in 10,000 μm^2 (NvC), granular diameter in nm (GD), volume density of secretory granules in μm^3 (VvG), numerical density of secretory granules in μm^3 (NvG).

Fig. 2. Rostral area of the adenohypophysis of a female lamb. Double immunostaining (AP-ABC) with anti-ovine GH (*arrows*) and anti-ovine PRL (*asterisks*) sera. Note the presence of SMTs (brownish-grey) containing both hormones (*arrowheads*) $\times 560$

the software Microm Image processing (Microm, Spain). Twenty immunoreactive cells of each animal were chosen randomly from the different sections separated from each other by $50\text{ }\mu\text{m}$, in order to determine the area and cellular diameter. Twenty micrographs of entire GH and PRL cells and five of SMT cells per group were analyzed at a final magnification $\times 14,800$, in order to determine the size of 20 secretory granules per hormone and cell and the numerical density (Nv) of the secretory granule in μm^3 . Nv was estimated according to the point-counting methods by WEIBEL and GÓMEZ (1962) in which $Nv = K/\beta \times Na^{3/2}/Vv^{1/2}$, Na being the number of secretory granules per unit of cytoplasmic area, Vv the density volume of the secretory granule, and K and β the constants ($K=1.05$ and $\beta=1.38$ for this study). Density of granular volume in μm^3 (Vv) was determined on photographs magnified $\times 11,500$, following WEIBEL's (1979) stereological method of area analysis, in which $Vv = Ao/At = Vo/Vt$; Ao corresponds to the total area of the profiles due to a particular secretory granule, whereas At is the total

area of the section, Vo is the volume occupied in the section and Vt is total volume of the section.

The statistical analysis was carried out with the program STATISTIX 3.5 (Analytical Software). Differences between means were determined by applying Scheffe's and Tukey's test. Significance is reported at the level of $P < 0.01$.

RESULTS

The parameters of GH and PRL cells are shown in Tables 1-2 and Figure 1. SMTs were found more frequently in the rostral areas, sometimes near to wide lumina capillaries, isolated or forming small groups. The cytoplasm showed a brownish-grey immunoreaction to the double-labeling technique, and its shape is rounded, elongated or polygonal (Fig. 2). In prepuberal animals, cell area ($57.55 \pm 14.41\text{ }\mu\text{m}^2$) was smaller than that of anoestrus ewes ($69.78 \pm 3.66\text{ }\mu\text{m}^2$). In lactation there were no significant differences in cell area, with values similar to those of

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Fig. 3. Double immunogold labelling of an adenohypophyseal section of an anoestrus ewe showing an SMT with unclear cellular limits. Note dilated cisternae of the endoplasmic reticulum (*ER*), GH granules (10 nm gold particles) and PRL granules (20 nm gold particles). $\times 14,000$. **Inset.** Higher magnification of the area enclosed in the rectangle. $\times 22,000$

Fig. 4. Double immunogold labelling of an adenohypophyseal section of a ewe in early lactation showing an isolated SMT. GH granules (19 nm gold particles, *arrowheads*) and PRL granules (20 nm gold particles, *arrows*). $\times 18,000$

ewes. Cell numerical density decreased with age, from 1.4 ± 0.03 in prepuberal females to 0.27 ± 0.01 in anoestrus ewes. During lactation there was a rise in the number of SMTs in early stages (0.7 ± 0.02); this value is lower than those of anoestrus ewes both in late lactation (0.17 ± 0.01) and after weaning (0.08 ± 0.01) (Fig. 1).

In all animals, typical GH and PRL cells with medium-sized and large secretory granules, respectively, were immunolabelled with different sizes of gold particles (Tables 1, 2). These two cell types, as well as SMT cells, vary in size and shape, with medium-sized secretory granules. The ultrastructural features of SMTs did not change during the different stages considered in this study, and when SMTs were found in groups, cell boundaries were unclear (Fig. 3). Isolated (Fig. 4) and grouped SMT cells were surrounded by monohormonal GH and PRL cells. The rough endoplasmic reticulum was moderately developed and arranged in narrow cisternae, occasionally revealing slight dilation, and evenly distributed throughout the cytoplasm (Fig. 3). The Golgi complex was poorly developed. Secretory granules exhibited immunoreaction to only one antiserum (Figs. 3-4). Significant differences in size and volume density ($P < 0.01$) were found between the secretory granules of the GH and PRL populations (Tables 1, 2). Granules immunolabelled with anti-GH serum were smaller (361 ± 82.5 nm) than those labeled with anti-PRL (427 ± 133.6). Volume density (V_v) of GH secretory granules (3.05 ± 0.49) was smaller than that of PRL (3.36 ± 0.50), whereas numerical density (N_v) was similar in both (GH = 0.23 ± 0.04 ; PRL = 0.29 ± 0.02). Degenerated cells or mitosis were never observed.

DISCUSSION

The use of light and electron microscopy employing the double immunolabeling clearly demonstrated the existence of bihormonal cells, the SMTs that contain both GH and PRL in prepuber females and ewes in anoestrus or at different stages of lactation, confirming that this cell type is routinely present in ovine adenohypophysis.

The population of SMTs is low compared to other cell types. These cells are difficult to be observed by electron microscopy because they are mostly found in rostral areas. For this reason the number of SMTs reported in this study is greater than that of THORPE et al. (1990) in sheep, whose counts were limited to sections immunolabelled with colloidal gold. Percentage values are similar to those described by SÁNCHEZ et al. (1994) for lactating goats, and lower than those

reported by FUMAGALLI and ZANINI (1985) and KINEMAN et al. (1991) for cattle.

The highest number of SMTs is found in prepuberal animals. This number decreases with age, although always in low proportions, as YAMAJI et al. (1992) reported for young and adult rats. Most prevalent at early lactation, SMTs gradually decrease in number through late lactation, as described by PORTER et al. (1990) in rats. The number of GH cells decreases with age, due to reduced hormonal demand in anoestrus ewes as shown by BERNABÉ et al. (1995-96b), and remains fairly constant during lactation. In contrast, PRL cells increase numerically with age, due to the existence of a cell population that is inactive (40%) (BERNABÉ et al., 1995-96a). This rise is most apparent during lactation, and thereafter decreases to values similar to those of anoestrus ewes after weaning (GÓMEZ-MARÍN, 1994). When the three cell types are viewed as a whole, increasing age produces a slight rise in the number of acidophilic cells, most apparent during lactation, and later decreases to values at those of anoestrus ewes after weaning. These changes may well correspond to the transformation of somatotrophs into lactotrophs induced by age and lactation, as suggested by PORTER et al. (1991). Degenerated cells were never observed.

Hormone storage pattern was akin to that of monohormonal cells. In small ruminants, GH and PRL possibly are stored in separate secretory granules, since they never show immunolabelling to both antisera in the same granule, only to one of them, in agreement with the data from SÁNCHEZ et al. (1994) in goats and HASHIMOTO et al. (1987) in cattle. The presence of PRL and GH in separate granules within the same cell can be explained as the result of a sorting process at the level of the Golgi complex (HASHIMOTO et al., 1987) or by the fusion of PRL and GH cells (TAKAHASHI, 1992). Different results have been reported by THORPE et al. (1990) for sheep, FUMAGALLI and ZANINI (1985) for cattle, and NIKITOVITCH-WINNER et al. (1987) and ISHIBASHI and SHIINO (1989) for rats. According to FUMAGALLI and ZANINI (1985), the granules with intermixed hormones may have originated from a merger of preassembled granules containing either GH or PRL. Ultrastructural features are not different from those of monohormonal cells, in agreement with PAPKA et al. (1986) and SÁNCHEZ et al. (1994), a conclusion confirmed by comparing the granule diameter of SMT and monohormonal cells. GH granules of SMTs are similar to those observed in the monohormonal cells of prepuberal females and ewes at various stages of lactation, but are smaller and significantly different from those of anoestrus ewes. PRL granules of SMTs are

similar to those found in the monohormonal PRL cells of lactating sheep but larger than those found in prepuberal females and ewes in anoestrus and after weaning, when a high percentage of cells seem to be inactive (GÓMEZ-MARÍN, 1994; BERNABÉ et al., 1995-96a). Granules immunolabelled with anti-GH serum are larger than PRL granules (ISHIBASHI and SHIINO, 1989), in contrast with the findings by SÁNCHEZ et al. (1994) for lactating goats and BASSETTI et al. (1989) for humans.

The functional significance of this cell type has yet to be established. SÁNCHEZ et al. (1994) and PASSOLI et al. (1994) claim that this population is distinct from GH and PRL cells, and unrelated to the physiologic state of the animal. The high number found in young females and at onset of lactation, together with the numerical variation of GH and PRL cells, suggest a second hypothesis, in which the SMTs are a transitional type which can change from GH to PRL via a bidirectional process, depending on functional state (PORTER et al., 1990, 1991). However, our results are not conclusive since the number of SMTs is always relatively low and the percentage of granules in one of the two hormones is similar at all stages without a noticeable change in GH production in prepuberal animals, or to PRL during lactation, as a great cell density might indicate. Further studies are therefore required to explain this finding. On the other hand, such a transformation cannot be ruled out because of the biochemical similarity of monohormonal cells, whose receptors and second messengers have been shown to be alike (KASHIO et al., 1990).

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