Histochemical Study of Follicles in the Senescent Porcine Pituitary Gland

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Summary. Follicles displaying a positive periodic acid-Schiff reaction to a colloid substance in the anterior pituitary gland have been observed in many vertebrates, including humans. It is also known that these follicles greatly increase in number and size with age. We therefore performed histochemical analysis of these colloid follicles in the senescent porcine pituitary gland in order to clarify their nature and biological significance. Results from various histological stains suggest that the colloid contains some polysaccharides or glycoproteins. In addition to the histological stains, lectin histochemistry revealed that the colloid contains sialic acid, N-acetyl galactosamine and galactose. Also, lectin staining indicated that some glycoproteins, most likely Asn-linked sialoglycoproteins, are localized in the colloid. The cells (FS cells) surrounding the colloid were densely stained with an antibody to S-100 protein but were not stained with antibodies to any other anterior pituitary hormones. Frequently mammotrophs (PRL cells), and occasionally gonadotrophs (LH cells) were found closely associated with folliculo-stellate cells (FS cells) which lay next to the large colloid containing follicles. This suggests that not only are the FS cells important in the production of the colloids, but the adjacent LH and PRL cells in some way also contribute to their formation.

Folliculo-stellate cells (FS cells) in the anterior pituitary gland were first described by Rinehart and Farquhar in 1953. FS cells were found to completely lack secretory granules and be further characterized by the presence of very long processes which surrounded adjacent glandular cells. The morphology of FS cells therefore suggests that they play an important role in the control of neighboring hormone secreting cells. Several functions have been proposed for FS cells. First, the abundance of lysosomes within them suggests that they may act as scavenger cells (Dingemans and Feltkamp, 1972; Vila-Porcile, 1973; Stokreef et al., 1986). In this connection, their pronounced phagocytic activity may have an important role in the removal of apoptotic cells (Drewett et al., 1993). Secondly, FS cells are known to secrete several growth factors such as fibroblast growth factor (FGF) (Ferrer et al., 1987), pituitary-derived vascular endothelial growth factor (PVEGF) (Gospodarowicz and Lau, 1987), follistatin and activin (Katayama et al., 1992; Kaiser et al., 1992) and interleukin-6 (IL-6) (Vankelcom et al., 1989, 1993). Thirdly, nitric oxide synthesis has been reported in FS cells (Ceccatelli et al., 1993). Also, one of the present authors (Inoue et al., 1992) has established a model cell line for FS cells (TtT/GF). These TtT/GF cells respond to the novel hypophysiotropic peptide, pituitary adenylate cyclase-activating peptide (PACAP), by increasing cAMP production, releasing IL-6, and increasing cell growth (Matsumoto et al., 1993). Interestingly, TtT/GF cells stimulate pituitary tumor growth in nude mice (Koyama et al., 1995). This result from the model cell line thus suggests that FS cells themselves secrete growth factor(s) which stimulate cell growth and proliferation through paracrine control. However, the putative tumor growth factor(s) produced by FS cells have yet to be identified.

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All of the above findings suggest that FS cells play an important role in the control of glandular cell function and proliferation, but the precise function of these FS cells remains under discussion. Following the first report by Kagayama (1965), follicle formation by FS cells in the anterior pituitary gland has been reported in many vertebrates, including humans. In normal animals these follicles contain some type of electron lucent colloidal substance. The contents of these follicles, however, change to an electron dense material in response to osmotic stress (Selye, 1943; Selve and Hall, 1943), adrenalectomy (Farquhar, 1957), gonadectomy (Ferrer, 1956; Dingemans and Feltkamp, 1972), thyrotropin-releasing hormone (TRHa, b), and L-dopa treatment (Harrison et al., 1982). Electron dense material also appears when pituitary tissue is implanted under the kidney capsule (Inoue et al., 1987). Interestingly, it has been reported that these follicles become more numerous with age in bats (Anthony and Gustafson, 1984), in humans (Ciocca et al., 1984), in guinea pigs (Kameda, 1991) and in pigs (Kubo et al., 1992). Also, a change in the size of the follicles and the formation of para-crystalline material in the follicular colloid have been reported in the bat during hibernation (Nunez and Gershon, 1982). It has been suggested that these follicles and the colloid produced within them by FS cells may yield important information about the function and purpose of the FS cells. Therefore, an analysis of the follicular colloid may shed light on the role and function of the colloid as well as the function of the FS cells which produce it. This may be particularly true in senescent animals due to the greater abundance of the colloid in this age group. To date there has been no chemical or histochemical analysis of the colloid produced and released into the follicles by these cells. In this paper we report the results of a histochemical analysis performed on the colloid found in the FS cells of the anterior pituitary gland.

MATERIALS AND METHODS

Light microscopy

Porcine pituitary glands from approximately 3-year-old aged animals and 6-month-old young animals obtained from slaughter houses were used in this study. For light microscopy, the pituitary glands were dissected into small pieces and fixed in either Bouin-Holland fixative or in 10% formalin for 24 h. They were subsequently dehydrated through ascending concentrations of ethanol. After clearing with xylene, the tissues were embedded in paraffin, and 5 µm thick paraffin sections were prepared for use in the histological and histochemical studies.

Histochemistry and immunohistochemistry

The tissue sections were first tested for the presence of carbohydrates by general procedures such as the PAS reaction, Bauer-Feulgen reaction or Best's carmine stain (Sheehan and Hrapchak, 1980). A variety of classical stains were then used to specifically identify various classes of carbohydrates and other substances: Alcian blue and iron diamine were used to detect acid glycoprotein; mucicarmine-rapid mucin staining was used to detect mucin; and amyloid material was identified using Congo red and an iodine reaction (Sheehan and Hrapchak, 1980). The Feulgen reaction was performed to demonstrate deoxyribonucleic acid (DNA) and the Schmorl method for lipofuscin (Sheehan and Hrapchak, 1980). Additionally, Sudan black B or Nile blue reactions for lipid were applied to frozen sections. Eight µm thick frozen sections were made with a cryostat (Cryocut E, Reichert-Jung). A test for acid phosphatase was carried out according to Barka and Anderson (1962). Methyl green or light green were used as counterstains.

Fig. 1. Double staining with the periodic acid Schiff reaction (PAS) and with immunocytochemistry for the S-100 protein (a-d), and with lectin histochemistry (e and f). a. A low magnification (×36) micrograph of the pars distalis (PD), pars intermedia (PI), pituitary cleft (PC) and the pars nervosa (PN). Many PAS positive follicles can be seen in the pars distalis as well as a few in the pars intermedia (arrow), but none are present in the pars nervosa. b. A highly magnified (×320) view of a well developed colloid follicle seen in Figure 1a. Note that some heterogeneous substance is present within the colloid (arrow). This well developed colloid is completely surrounded by several S-100 positive folliculo-stellate cells. c. Four small adjacent colloid follicles (arrow) coalescing to make one large follicle in the pars distalis can be seen. ×320. d. A highly magnified (×320) view of a colloid in the pars intermedia which is seen in Figure 1a (arrow). Colloid follicles in the pars intermedia are also surrounded by S-100 protein positive cells as in the pars distalis. e and f. Positive staining by lectin histochemistry of well developed colloid in the senile porcine pituitary gland using PHA-L4 (e) and MAM (f) respectively. Note how the small follicular lumens are also stained with these lectins (arrows). Parts of the cytoplasm of folliculo-stellate cells are positive for MAM (f). e, f: ×230
Fig. 1. Legend on the opposite page.
In addition to the above classic histological stains, some tissue sections were immunocytochemically stained using antisera for S-100 protein (DAKO-A/S, Denmark) and antisera for anterior pituitary hormones, i.e., growth hormone (GH, HAC-RT25-02RBP85), prolactin (PRL, HAC-RT25-01 RBP85), luteinizing hormone (LH, NIH, NIAMDD, AFP-2-11-27), thyroid stimulating hormone (TSH, HAC-RT29-01RBP86) and adrenocorticotropic hormone (ACTH) which had been raised from porcine ACTH (INOUE and HAGINO, 1984). Working dilutions of the antibodies used in the immunocytochemical tests were as follows: 1:1000 for anti-GH, ACTH, and S-100 protein; 1:2000 for anti-PRL and anti-LH; and 1:5000 for anti-TSH. The specificity of all of these antibodies had been tasted by an absorption control. After applying the primary antibodies for 24 h, the sections were stained by the peroxidase-anti-peroxidase method by STERNBERGER et al. (1970). This was accomplished by first deparaffinizing the sections and pretreating them with 0.5% sodium metaperiodate for 5 min. Following this, they were treated with 1% bovine serum albumin to block nonspecific reactions. After incubating with primary antibodies for 24 h, the sections were next incubated with goat anti-rabbit IgG (HAC-RBA2-04GTP86) at a dilution of 1:1000 for 1 h. The sections were then incubated with the PAP complex (PAP; DAKO-A/S, Denmark) at a dilution of 1:200 for 1 h. Finally, they were visualized with a diaminobenzidine (DAB) solution.

**Electron microscopy**

For general electron microscopic observation, the pituitary glands were dissected into small pieces and fixed with 2.5% glutaraldehyde followed by post-fixation in 1% osmium tetroxide. After fixation, the tissues were dehydrated with ethanol, treated with propylene oxide, and embedded in Epon-Araldite in accordance with conventional methods.

Carbohydrates were detected by the periodic-acid-silver methenamine (PAM) method according to RAMBOURG (1967). Briefly, tissue was fixed with 2.5% glutaraldehyde, dehydrated without post-fixation in 1% osmium tetroxide, and embedded in Epon-Araldite. Ultrathin sections mounted on nickel grids were pretreated with 1% metaperiodate for 30 min to oxidize the glucose base. This was followed by reacting the sections for 30 min in a silver methenamine solution at 60°C.

Lectin staining was accomplished by first fixing the tissues in a mixture of 0.5% glutaraldehyde and 4% paraformaldehyde for 24 h. Next, the tissue was post-fixed in reduced osmium for 30 min. The reduced osmium was prepared by adding a 1.5% potassium ferrocyanide to a 1% osmium tetroxide solution (TAMAKI and YAMASHINA, 1994). After dehydration, the tissues were embedded in Epon-Araldite in accordance with conventional electron microscopy. Staining was accomplished by incubating the ultrathin sections with biotinylated lectins for 24 h. After the lectin application, the sections were incubated for 1 h with a streptavidin-labeled colloidal gold (Zymed Lab. Inc., USA) at a dilution of 1:50. Additionally, semithin sections were also made for lectin staining for light microscopy. These sections were mounted on glass slides and treated with ethanol-saturated KOH to remove the epoxy-resin (LANE and EUROPA, 1965). They were then subjected to lectin staining as described above.
RESULTS

General light microscopic histochemistry

A large number of follicles containing a PAS positive colloidal substance were observed in the aged porcine pituitary glands (Fig. 1a). The mean diameter of these follicles, which were mainly present in the periphery of the anterior pituitary gland, was found to be 21.3±7.2 μm. A small number of follicles were also localized in the intermediate lobe in these animals (Fig. 1d). Interestingly, the relationship between the weight of the pituitaries in old and young animals was reflected in the amount of colloid present. The wet weight of the pituitary glands in aged animals was about 5 times greater (1.05±0.07 g) than that in young animals (0.20±0.01 g), a relationship which was approximately proportional to the amount of colloid in the pituitaries of the two age groups.

Several classic histochemical stains were used in the analysis of the colloidal substance, with results shown in Table 1. As can be seen, the colloid positively stained with methyl green, light green, periodic-acid-silver methenamine (PAM), Bauer-Feulgen reaction, and Best's carmine, but failed to stain with Alcian blue for acid glycoproteins, and Congo red and the iodine reaction for amyloid. Also, lipids were not present (Sudan black B and Nile blue), nor was mucin (mucicarmine and rapid mucin). Acid phosphatase was negative and there was no indication of lipofuscin by means of the Schmorl method. The Feulgen reaction showed that no DNA was present. Thus, these results suggest that some glycoproteins or polysaccharides are present in the colloid. However, the colloid is probably not rich in sulfate or carbonate base due to the negative results obtained with Alcian blue and the iron diamine test. It was also clear that the colloid does not contain mucin since it did not stain with mucicarmine or the rapid mucin staining method. Also, the colloid was stable in the presence

Table 1. The histological stainings of colloid substance in the pituitary follicles.

<table>
<thead>
<tr>
<th>Positive Reaction</th>
<th>Negative Staining</th>
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<tbody>
<tr>
<td>PAS reaction</td>
<td>Alcian blue</td>
</tr>
<tr>
<td>Best's carmine</td>
<td>Iron diamine method</td>
</tr>
<tr>
<td>Bauer-Feulgen reaction</td>
<td>Feulgen reaction</td>
</tr>
<tr>
<td>PAM</td>
<td>Sudan black B</td>
</tr>
<tr>
<td>Methyl green</td>
<td>Nile blue</td>
</tr>
<tr>
<td>Light green</td>
<td>Iodine reaction</td>
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<td></td>
<td>Congo red</td>
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<tr>
<td></td>
<td>Acid phosphatase</td>
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<tr>
<td></td>
<td>Schmorl method</td>
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<tr>
<td></td>
<td>Mucicarmine</td>
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<td>Rapid mucin</td>
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PAM: periodic-acid-silver methenamine

Table 2. Results of histochemical stainings of sugar moieties in the pituitary colloid using various lectins.

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Specific binding sites</th>
<th>Intensity of staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A</td>
<td>Mannose, aMG, aMM</td>
<td>−</td>
</tr>
<tr>
<td>ABA</td>
<td>Galβ 1-3GalNAc</td>
<td>+</td>
</tr>
<tr>
<td>DSA</td>
<td>N-acetyl lactosamine</td>
<td>−</td>
</tr>
<tr>
<td>Lotus</td>
<td>L-Fucose</td>
<td>−</td>
</tr>
<tr>
<td>WGA</td>
<td>GlcNAc</td>
<td>−</td>
</tr>
<tr>
<td>SBA</td>
<td>GalNAc</td>
<td>−</td>
</tr>
<tr>
<td>PHA-E4</td>
<td>GalNAc</td>
<td>−</td>
</tr>
<tr>
<td>PHA-L4</td>
<td>GalNAc</td>
<td>++</td>
</tr>
<tr>
<td>RCA 60 (Ricin)</td>
<td>β-Gal, Lactose</td>
<td>++</td>
</tr>
<tr>
<td>MAM</td>
<td>Siaα2-3Gal</td>
<td>++</td>
</tr>
<tr>
<td>SSA</td>
<td>Siaα2-6Gal</td>
<td>+</td>
</tr>
</tbody>
</table>

of chondroitinase, hyaluronidase, neuraminidase, collagenase and DNase, but was labile when exposed to an alkaline solution or proteinase K (data not shown). This suggested that the colloid contained some glycoproteins in its matrix.

Several types of lectins were used in an attempt to stain specific sugar moieties. Table 2 shows the colloid was strongly stained by MAM (Fig. 1f), RCA 60 and PHA-L4 (Fig. 1e), but only weakly by ABA and SSA. However, no staining at all occurred with Con A, DSA, Lotus, PHA-E4, SBA and WGA. These results imply that the colloid contains sialic acid, N-acetyl galactosamine and galactose. No anterior pituitary hormones such as LH, GH, PRL, ACTH, or

Fig. 2. Immunocytochemistry of anterior pituitary hormones. Results of staining for PRL (a and b), LH (c), GH(d), ACTH (e) and TSH (f) can be seen. Many of the prolactin cells (a and b) and some of the LH cells (e) closely associate with the colloid-containing folliculo-stellate cells. However, the other pituitary hormone-secreting cells show no such association and are randomly distributed within the field of view. a: x50; b and c: x120, d: x200, e and f: x180
TSH could be detected in the follicles by immunocytochemistry (Fig. 2).

Most of the colloid follicles, especially the small follicles, were completely surrounded by S-100 protein positive FS cells (Fig. 1c). Large follicles were also surrounded by S-100 positive cells, but in some cases only by a very thin strip of cytoplasm (Fig. 1b). Most of the other hormone secreting cells of the pituitary, such as GH cells, ACTH cells, and TSH cells, were not in any way associated with the FS cells that surrounded the colloid (Fig. 2d-f). In contrast, the PRL cells and LH cells, especially the PRL cells, were closely associated with the FS cells which surrounded the colloid (Fig. 2a-c).

Electron microscopic observations

The electron microscopic observations generally agreed well with results obtained by light microscopy. The colloid follicles appeared completely surrounded by FS cells. The FS cells which surrounded the large colloid follicle did so with an extremely thin, narrow band of cytoplasm (Fig. 3). The FS cells themselves contained an abundance of intermediate filaments and had well developed tight junctions (Fig. 3, 4a, 5). A few microvilli were observed on their luminal surface. Several sizes of follicle were present in the senile porcine pituitary glands. Some of the small colloid follicles contained a heterogeneous substance which was embedded in a homogeneously electron dense matrix (Fig. 4a). However, in contrast to the small follicles, the well developed colloid follicles were quite homogeneous (Fig. 3). In rare cases, however, secretory cells were observed within extremely well developed follicular lumens which in turn were themselves surrounded by a very thin layer of FS cells cytoplasm. The membrane structure of these embedded secretory cells was not distinct; possibly these cells were necrotic cells which in some way became included in the follicle. Immunocytochemical tests performed with antibodies to anterior pituitary hormones did not detect any hormones within these entrapped secretory cell fragments.

The silver methenamine method for carbohydrates strongly stained the follicular colloid. Some strongly staining granules and lysosomes were observed within the FS cells. Additionally, some small round to rod-like granules in the cytoplasm of FS cell were also stained by the silver methenamine technique (Fig. 4b). In agreement with the light microscopic results, lectin staining at the electron microscopic level showed a strong positive reaction for MAM (Fig. 5b), RCA60 (Fig. 5a) and PHA-L4 in the colloid.
Fig. 4. Electron micrographs of small follicles which are surrounded by folliculo-stellate cells in the pars distalis of the senile porcine pituitary gland. a. Small follicles containing a heterogeneous substance (arrow) in their lumen can be seen. The follicle is completely surrounded by folliculo-stellate cells (FS) which possess well developed tight junctions (J). N nucleus of the granular cell. ×7,000. b. The histochemical demonstration of carbohydrate by the silver methenamine method. The colloid substance (C) and some rod-like cytoplasmic granules (arrowheads) are positively stained by this procedure. ×32,000
DISCUSSION

A number of reports have appeared which state that pituitary glands in senescent animals contain numerous follicles which include within them a dense colloidal substance. Such follicles have been described in the pituitaries of guinea pigs (KAMEDA, 1990, 1991), bats (ANTHONY and GUSTAFSON, 1984; NUNEZ and GERSHON, 1982), cattle (BASSETT, 1950), pigs (KUBO et al., 1992), and humans (OSAMURA and WATANABE, 1978; CIOCCA et al., 1984). The presence of these numerous follicles in senescent porcine pituitary glands has been confirmed in the present paper. Double staining with PAS and with an antibody to S-100 protein has clearly shown that the colloid was PAS positive and was completely surrounded by FS cells. It appears that the presence and the number of follicles in the senile animal may be a reflection of a change in the function of the FS cells themselves with age. For this reason, the importance of an analysis of colloid formation and the substance from which it is formed becomes apparent. Until now, however, no systematic histochemical study of the substance comprising the colloid has been carried out.

In the present study, we found the wet weight of senescent pituitary glands to be about five times greater than that of young animals. This is in agreement with the proportion of the colloid content found in the old and young groups. The altered appearance of the pituitary glands including colloid formation in senescent animals may be related to increased prolactin and gonadotropin levels in post-menopausal animals (SHARR et al., 1975). It is noteworthy that many lactotrophs, and to a smaller extent gonadotrophs, are clustered around the colloid-containing FS cells in the senescent porcine anterior pituitary gland. This phenomenon implies a relationship between the FS cells and the lactotrophs or gonadotrophs associated with them. That is, some of the hormone secreting cells may be regulated by the FS cells. Although we have no data showing how colloid containing FS cells regulate lactotrophs and gonadotrophs, one possibility is that, in the aging porcine pituitary gland, the FS cells regulate them by secreting growth factors, or by altering their microenvironment, such as their extracellular matrix.

Our electron microscopic observations have shown that the follicles occur in varying sizes in the senescent porcine pituitary gland, in good agreement with

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**Fig. 5.** Histochemical detection of sugar moieties using RCA 60 (a) and MAM (b). The colloid stained positively with colloidal gold (arrows) labeled RCA 60 and MAM. Arrowheads show junctional complex of folliculo-stellate cells. FS folliculo-stellate cells. ×33,000.
a previous paper by Kamed (1991). Since the follicles were completely surrounded by FS cells, it leads one to conclude that the major part of the colloid was produced by these FS cells. Some of the follicles contained a heterogeneous substance in a homogeneous electron dense matrix. Similar structures have been reported by Vila-Porcile (1973), who suggested that these heterogeneous substances might be the products of the phagocytosis of degenerated glandular cells which have accumulated as residual bodies within the colloid. This interpretation is supported by Drewett et al. (1993), who reported that FS cells phagocytosed apoptotic prolactin cells which appear after the suspension of estrogen treatment, or which appear after the administration of dopamine agonists such as bromocriptine. Although we have failed to identify the cell types by immunocytochemistry, we too have found hormone secreting cells within the follicular lumen. It is possible that these cells might have entered the follicular lumen after passing through the FS cells. Thus some components of the follicle may be the result of phagocytosis, and stored as residual bodies within the follicle. As mentioned above, it seems likely that the homogeneous matrix in the follicles is produced by FS cells. Although we have no data to prove that the FS cell produces the matrix of colloid follicles, the observation of small round and rod-shaped silver methenamine positive granules in the cytoplasm of the FS cells is of interest, as it suggests that the colloidal substance may be first produced by the FS cells and subsequently secreted by it into the follicular lumen. This possibility is supported by the finding that lectin staining with MAM, which is specifically bound to sialic acid, stains positively not only to the colloid, but also with the cytoplasm of the FS cells.

We found that the colloid developed in the senescent porcine pituitary gland strongly stained both with PAS and with silver methenamine at the light and electron microscopic levels. This clearly indicated that the colloid contained carbohydrates. Since the colloid could be digested by protease K, the presence of protein was also demonstrated. This protein is probably a glycoprotein. The colloid was found to stain with MAM, PHA-L4 and RCA 60. This indicated that the colloid contained sialic acid, N-acetyl galactosamine and a galactose rich glycoprotein. Previously the relationship between the sugar chain structure of glycoproteins and the lectin reaction was systematically studied by Cummings and Kornfeld (1982). According to their report the glycoprotein in the pituitary colloid may be conjugated with some asparagine-linked oligosaccharides. Presently, an attempt to purify the colloidal and its associated substances is under active investigation in our laboratory.

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