Ultrastructural and histochemical investigations of Call–Exner bodies in rabbit Graafian follicles

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Summary. In a histological survey of 19 mammalian species, Call–Exner bodies of conventional size and appearance were found in only 5, namely, human, rhesus monkey, rabbit, guinea-pig and sheep. Rabbit ovaries were used for characterizing these bodies using quantitative histochemistry, lectin binding and electron microscopy. Call–Exner bodies were topographically distinct lacunae of the extracellular space probably containing hyaluronic acid with proteoglycan complexes. The staining characteristics of the antrum and Call–Exner bodies were generally similar. However, in contrast to the antrum, the smaller lacunae contained suspended filaments with a distinctive peripheral membrane upon which a rosette of granulosa cells was resting. The membrane and narrow intercellular clefts probably prevent much exchange of large glycosaminoglycan complexes with the antrum. The origin and significance of Call–Exner bodies require further study, but it is clear that they are associated with secretion rather than with necrosis as sometimes suggested.

Keywords: Call–Exner bodies; glycosaminoglycans; Graafian follicle; granulosa cell; lectin; rabbit

Introduction

In 1875 Call & Exner described new cellular structures (“Zellen”) within the epithelial layer of the rabbit follicle. They tentatively equated them with newly formed oocytes because of resemblance to primordial follicles. While the mistaken identity was soon apparent, the character of the so-called bodies of Call and Exner has been in dispute ever since. It has been suggested that the bodies are sites of liquefaction following necrosis of granulosa cells (Thompson, 1919), a view which has been widely held to the present day. On the basis of ultrastructural studies of mouse follicles, Hadek (1963) attributed them to secretory droplets forming in the Golgi apparatus of granulosa cells, although the small size of the droplets led Mossman & Duke (1973) to question whether he was in fact studying the same structures. Honoré (1899–1900) suggested that the bodies are extracellular sites of accumulating secretory products. This view was endorsed by both Robinson (1918) and Motta (1965) who regarded them as dispersed parts of the follicular antrum.

In view of this confusion and the existence of only rudimentary histochemical information, the character of Call–Exner bodies has been investigated as part of more extensive studies of the extracellular environment of follicle cells. Since they are not present in all mammals (Mossman & Duke, 1973; Motta, 1967) it was necessary to identify a suitable animal model before starting histochemical and ultrastructural studies.

Materials and Methods

The occurrence of Call–Exner bodies was investigated in 19 species by examining serial histological sections prepared for an earlier study from at least 4 young individuals (see Gosden & Telfer, 1987). The rabbit ovary was chosen for

further study because discrete Call–Exner bodies were always found in Graafian follicles and additional fresh tissues were available.

Ovaries from young oestrous does were fixed either by direct immersion in fixative following dissection and slicing into fragments or by perfusion during anaesthesia with ethyl carbamate (1.75 g/kg, i.v.). Tissues for light microscopy were either prepared in aqueous Bouin's fixative with subsequent sectioning at 5 µm in paraffin wax or were fixed in formal–calcium fixative and sectioned at 12 µm in a cryostat.

Wax sections were stained for the periodic acid–Schiff (PAS) reaction or, for demonstrating carboxylated and sulphated glycosaminoglycans (GAGs), with the cationic dye Alcian blue (1% w/v at pH 2.5 in acetic acid or pH 1.0 in hydrochloric acid) (Lev & Spicer, 1964). Dewaxed sections were set aside for quantitative histochemistry. They were preincubated with ovine testicular hyaluronidase (170 U/ml: Sigma, London, UK) or chondroitinase ABC (1.7 U/ml: Sigma) in an humidified chamber for 3 h at 37°C (Delgado & Zoller, 1987) and mounted for microscopy. Optical densities of the Call–Exner bodies stained with Alcian blue at pH 2.5 were measured at 610 nm using a Vickers M86 scanning and integrating microdensitometer. Oil immersion optics were used with a total magnification of $\times 500$ and a mask size (A6) slightly larger than the maximum diameter of the Call–Exner bodies (approximately 30 µm). A further series of experiments was carried out to compare digestion by mammalian hyaluronidase with that by fungal hyaluronidase from *Streptomyces hyalurolyticus* since the latter is more specific (Yamada, 1973).

Carbohydrates in the Call–Exner bodies were characterized further using lectins labelled with tetramethyl-rhodamine–isothiocyanate (TRITC) which were either conjugated in the laboratory or prepared commercially (Sigma, London). Dewaxed paraffin sections and cryostat sections were used to investigate binding from a panel of 7 lectins (see Table 2), with working concentrations ranging from 0.1–1.5 mg/ml PBS. Sections were incubated in a lectin solution for 1 h at 37°C, either with or without an inhibitory sugar present (0.2 M) (Debray *et al.*, 1981; Lis & Sharon, 1986). They were washed in PBS and mounted in PBS–glycerol (1:1, v/v) containing 0.1 µg/ml of a DNA-binding fluorochrome (Hoechst 33342; Aldrich Chemical Co., Gillingham, Dorset, UK). Specimens were examined by episcopic fluorescence using a Nikon Labophot microscope with differential excitation and barrier filters for the two stains (filter types G and UV, respectively).

Specimens for electron microscopy were fixed in 1% glutaraldehyde for 1 h followed by a further hour in 1% osmium tetroxide in 0.13 M–Pipes buffer (pH 7.0): 15% picric acid was added to the fixative of half of them (aqueous, saturated solution). Dehydrated tissue was embedded in LR White resin (London Resin Company, London, UK) and thin sections were cut with a Porter–Blum MT2 microtome using glass knives. Sections showing a silver–white interference colour were mounted on single-hole type-1000 grids which were covered with Pioloform support film. Sections were stained with aqueous uranyl acetate followed by lead citrate (Reynolds, 1963).

Results

Call–Exner bodies of conventional size and appearance were clearly recognizable in Graafian follicles of human, rhesus monkey, rabbit, guinea-pig and sheep ovaries. They were absent in the other species examined (common shrew, hedgehog, pipistrelle bat, house and wood mouse, bank and field voles, Norway rat, domestic cat and dog, pigs, cattle, horse and common marmoset).

The Call–Exner bodies of rabbits varied in size from the limits of resolution of the light microscope (0.2 µm) to about 30 µm diameter. Several bodies were usually recognizable in a single follicle section at low magnification ($\times 100$) but counting was unreliable because of difficulties identifying the smallest bodies. They were represented in the largest multilaminar preantral follicles as well as in all Graafian types. The bodies persisted during early stages of atresia, but were absent in corpora lutea of two pregnant animals.

Each Call–Exner body was approximately spherical and enclosed by a rosette of granulosa cells that appear slightly columnar in wax sections, although more flattened in electron micrographs. They were located in both mural and stalk regions of the granulosa cell population and occasionally in the cumulus oophorus. They were eosinophilic and presented a reticulated appearance with a peripheral membrane when stained with PAS or Alcian blue (Fig. 1). Hyaluronidase significantly reduced Alcian blue staining (Table 1) and similar results were obtained irrespective of whether mammalian or fungal enzyme was used (37.6 and 34.6% reduction in optical density, respectively). Chondroitinase ABC also had a significant, if less marked, effect on staining. While the cumulative reduction in optical density was not tested, the sum of the independent effects of hyaluronidase and chondroitinase amounted to only about 50%. Satisfactory measurements of the antrum could not be made because of the irregular shape of this space and uneven distribution of stained material. Nevertheless, it was apparent that staining of the antrum diminished in larger follicles whereas that of the Call–Exner bodies did not vary.

Fig. 1. Rabbit Graafian follicle stained with Alcian blue to demonstrate glycosaminoglycans in the antrum and Call-Exner spaces (arrows). Nuclei have been counterstained with neutral red. $\times 160$.

Fig. 2. Small antral and multilaminar preantral follicles stained with TRITC-labelled wheat germ agglutinin. While background fluorescence is moderately high, the staining of the antrum, Call-Exner bodies (arrowed), basement membrane and zona pellucida of larger follicles is intense. $\times 85$.

Fig. 3. Two Call-Exner spaces are visible in this formalin-fixed frozen section which was stained with TRITC-labelled wheat germ agglutinin. The extracellular material and granulosa cells throughout the follicle show affinity for the lectin. $\times 340$.

Fig. 4. The same specimen as in Fig. 3 is visualized by staining with a DNA fluorochrome (Hoechst 33342). The Call-Exner spaces are unstained, indicating that they are not sites of cellular necrosis. $\times 340$.

Fig. 5. Call-Exner bodies prepared with Bouin's fixative and paraffin wax stain more extensively with wheat germ agglutinin than do formalin-fixed frozen sections (cf. Fig. 3). $\times 340$.

Fig. 6. The affinity of Call-Exner bodies (C) and antrum (A) for TRITC-labelled concanavalin A shown here is less than that for wheat germ agglutinin. $\times 340$.

Table 1. Measurements of Alcian blue staining intensity in Call–Exner bodies of rabbit Graafian follicles treated with hyaluronidase or chondroitinase

Group	Optical density (arbitrary units)*	% Reduction in optical density
Control	114.7 ± 4.2 (40)	
Hyaluronidase	71.6 ± 4.2 (31)	37.6
Chondroitinase	95.8 ± 5.0 (31)	16.5

*Mean ± s.e.m. (number of observations). The density was significantly reduced in both enzyme-treated groups compared with controls ($P < 0.001$, Student's *t* test).

Table 2. Relative binding of TRITC-labelled lectins to carbohydrates in the Call–Exner bodies and antral spaces of Graafian follicles in the rabbit ovary

Lectin	Specific inhibitory monosaccharide(s)	Call–Exner body	Antrum
<i>Phaseolus vulgaris</i> (red kidney bean)	—	—	+
<i>Arachis hypogaea</i> (peanut)	D-Galactose	±	+
<i>Phytolacca americana</i> (pokeweed)	N-acetyl-D-glucosamine	±	±
<i>Triticum vulgaris</i> (wheat germ)	N-acetyl-D-glucosamine	+++	+++
<i>Canavalia ensiformis</i> (Concanavalin A, jack bean)	N-acetyl-D-glucosamine, D-glucose, sucrose, D-mannose	+	+
<i>Ulex europaeus</i> (gorse)	L-Fucose	±	+
<i>Glycine max</i> (soybean)	N-acetyl-D-galactosamine, D-galactose	±	±

Despite widespread affinity of plant lectins for ovarian tissue there was some differential staining. While small preantral follicles had little affinity for any of the 7 lectins, the surface epithelium and cortex of the ovary stained with them all, and with high intensity in some cases (Table 2). Call–Exner bodies avidly bound wheat germ agglutinin (WGA) (Figs 2–5) and were stained weakly or moderately by other lectins (e.g. concanavalin A) (Fig. 6). WGA also stained precipitated material in the antrum, all granulosa cells of large follicles, follicle basement membrane and, in particular, the zona pellucida. Fluorescence was inhibited specifically by N-acetyl glucosamine and by predigestion with either of the hyaluronidases. The fluorescence in frozen tissue sections was less than in paraffin-wax sections, suggesting that formalin had stabilized less of the GAGs; on the other hand, nuclear fluorescence from H33342 staining was partly quenched in wax sections fixed with Bouin's fluid. DNA fluorescence was absent from the cavities of Call–Exner bodies.

The ultrastructural appearance provided no indications of cell death, nor did it reveal morphological distinctions between granulosa cells of the rosette and those elsewhere. A peripheral membrane bounded the cavity and, although reminiscent of the follicular basement membrane, was considerably thicker (30–50 nm with normal fixation, 45–75 nm when picric acid was added) (Figs 7–10). Filamentous material with a 'bottle brush' appearance was frequently, although not always (Fig. 9), present and sometimes presented an extensively folded reticulum (Fig. 7). The membrane was a consistently reliable feature for distinguishing Call–Exner bodies from other

Fig. 7. A Call-Exner body of a rabbit Graafian follicle. This approximately spherical body contains a reticulum of filamentous material bounded by a membrane. The filaments and membrane are absent from the antrum and other interstitial spaces. Glutaraldehyde and osmium. $\times 4600$.

Fig. 8. The specimen in Fig. 7 is viewed at higher magnification to show details of the filamentous material which appears to consist of densely branching macromolecules. The suspended filaments and membrane have a similar appearance. $\times 26\,000$.

Fig. 9. Smaller Call-Exner bodies often possess fewer suspended filaments, but a membrane is always present and can be considered as diagnostic of all such bodies. Long processes are frequently present (arrow). This specimen, which was fixed in the presence of picric acid, shows electron dense granules (probably proteoglycans) associated with the membrane. Glutaraldehyde and osmium. $\times 13\,000$.

Fig. 10. This specimen, which was prepared by the same method as in Fig. 9, demonstrates granules (12–20 nm diameter) at higher magnification. They are evenly distributed on the filaments and on the inner border of the membrane (arrow). $\times 26\,000$.

extracellular spaces. Globular electron-dense matter was associated with the filaments and membrane when treated with picric acid (Figs 9, 10). These filaments and membrane, which were similar in appearance, were absent from the antrum and other interstitial spaces of the follicle (Fig. 7). Apart from the membrane, there was no physical barrier between the various extracellular spaces since, except at occasional points of contact, granulosa cells were separated from one another by continuous intercellular clefts. Microvilli were absent, although larger processes were frequently present beneath the membrane (Fig. 9).

Discussion

The present findings concur with conclusions reached many years ago by Honoré (1899–1900), Robinson (1918) and Motta (1965) who pointed out that Call–Exner bodies are probably extensions of, and contiguous with, fluid in the antrum. Other experiments have shown that a marker of extracellular space, horseradish peroxidase, can permeate from the antrum between granulosa cells and into the cavity of Call–Exner bodies (R. G. Gosden, unpublished). Some workers have concluded that the bodies represent sites at which primordial follicles have become caught up in the development of a neighbouring follicle or represent localized sites of necrosis, but the absence of bodies in small preantral follicles and lack of any evidence of residual cellular organelles or DNA argue against these explanations. The Call–Exner bodies appear to be sites at which locally secreted macromolecules are accumulating. The character of carbohydrate moieties of proteoglycan/GAG complexes present can be tentatively inferred from histochemistry.

Since the bodies are PAS positive it has been assumed that they contain ‘glycoprotein’, although this is the extent of earlier histochemical studies (McKay *et al.*, 1961). Enzyme digestion combined with Alcian blue staining as a general but quantitative indicator of GAGs indicates that hyaluronic acid is a major constituent. The filaments presenting a ‘bottle brush’ appearance by electron microscopy are consistent with this conclusion, as are the results of staining with wheat germ agglutinin. This lectin is known to bind *N*-acetyl glucosamine sequences in internal chain positions (Goldstein *et al.*, 1975) and terminal sialic acid residues (Bhavanandan & Katlic, 1979), although interactions with cell surface glycoconjugates are complex and require cautious interpretation (Debray *et al.*, 1981). Extracellular glycoproteins such as fibronectin, which is known to be produced by these cells, could account for some of the residual staining after treating sections with hyaluronidase (Mera & Davies, 1985). Intense staining of the rabbit zona pellucida by WGA is not unexpected in view of the known oligosaccharide linkages with sialylation in the zonae of mice and pigs (Wassarman, 1988). Staining with WGA is also consistent with the presence of heparan sulphate, and the results can be generally taken to indicate that proteoglycans are also present. A number of studies of rabbit and mouse ovaries have shown that hyaluronic acid and chondroitin sulphate are major constituents of follicular fluid (Odeblad, 1954; Zachariae, 1957; Tadano & Yamada, 1978; Fowler & Guttridge, 1987), and quantitative biochemical studies show that chondroitin sulphate is secreted by granulosa cells into the antrum of some species whereas hyaluronic acid is primarily produced by the cumulus oophorus (Ax *et al.*, 1985). The present results cannot be used to draw inferences about the relative quantities of GAGs present because labelling by different lectins and the binding of Alcian blue to different GAGs is not equimolar. Diminution of staining after treatment with chondroitinase ABC does not necessarily indicate chondroitin sulphate-proteoglycans because this enzyme can also digest hyaluronic acid (Yamagata *et al.*, 1968; Yamada, 1974). If chondroitin sulphate was abundant, however, staining with soybean lectin would be expected to be more intense. The various studies indicate, however, that while the organization of macromolecular complexes differ the composition of the fluids in Call–Exner body and antrum are similar in respect of GAGs.

There are several possible reasons why there have been few references to Call–Exner bodies in recent years. The first is connected with the absence of the bodies in myomorph rodents which

replaced rabbits as major laboratory models for experimental reproductive physiology many years ago. The second is the practical difficulty of isolating the small, scattered droplets for biochemical analysis. Most of the limited attention on Call-Exner bodies is due to their value as histopathological aids for diagnosing human granulosa cell tumours (Novak & Woodruff, 1967). While histochemistry can provide preliminary characterization, experimental models are now needed to investigate the formation and distinction of Call-Exner bodies from the rest of the extracellular space. In-vitro models provide a potential means of progress because Harrison (1961) mentioned that cultured rabbit granulosa cells form hollow vesicles varying in size with gonadotrophic hormone stimulation and C. Torrance & R. G. Gosden (unpublished) have observed GAGs within unilaminar vesicles of sheep granulosa cells in collagen gel cultures. One of the major questions for future attention is why Call-Exner bodies undergo limited expansion while the protoantral cavities, which form at the same time, undergo continuous growth. This may lead to information about the control of secretion in the follicle which may influence granulosa cell adhesiveness and the architecture of intercellular spaces.

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