The appearance of glycoconjugates associated with cortical granule release during mouse fertilization

S. H. LEE, K. K. AHUJA, D. J. GILBURT and D. G. WHITTINGHAM

MRC Experimental Embryology and Teratology Unit, Woodmansterne Road, Carshalton, Surrey, SM5 4EF UK

Summary

For the first time, we have shown with appropriately labelled lectins that fucosyl- and sialyl-rich glycoconjugates are released into the perivitelline space of the mouse oocyte after activation by the fertilizing spermatozoon or artificial activation by the calcium ionophore A23187 or ethanol. The glycoconjugates show a punctate distribution over the oocyte surface except for the microvilli-free area overlying the second meiotic spindle from which they are absent. Their appearance in the perivitelline space is associated with the release of the cortical granules suggesting that they represent part of the cortical granule exudate. Soon after the glycoconjugates appear, they begin to aggregate. The process continues until the beginning of cytokinesis at first cleavage when a single large aggregate is found within the cleavage furrow. Most of the labelled glycoconjugates disappear by the late 2-cell stage and no evidence was found for their presence during the later preimplantation period. This technique is suitable for monitoring the kinetics of the cortical reaction in mammalian oocytes and investigating the importance of the glycoconjugates in early preimplantation period.

Key words: surface glycoconjugate, exocytosis, polyspermy, fertilization, mammal, oocyte.

Introduction

The completion of meiosis and the release of cortical granules (CG) are two of the earliest responses observed during the process of mammalian oocyte activation induced by entry of a fertilizing spermatozoon (Austin, 1961) or by artificial activating agents (Whittingham, 1980). The CG material released into the perivitelline space (PVS) modifies the properties of the oocyte plasma membrane and the overlying acellular zona pellucida to prevent the entry of further spermatozoa into the oocyte, the so-called 'block to polyspermy' (Austin, 1956; Barros & Yanagimachi, 1971; Gwatkin et al. 1973). The biochemical nature of CG material and the molecular mechanisms whereby the block to polyspermy is achieved are not fully understood.

Previous electron microscopic studies have shown that CG exocytosis involves a close apposition between CG and oocyte plasma membrane followed by fusion of the membranes and release of CG material into the PVS (Szollosi, 1967; Nicosia et al. 1977). As a result, the CG membranes contribute to the plasma membrane of the newly penetrated oocyte. The material released into the PVS is thought to contain enzymes which are short-lived and disperse rapidly modifying the properties of the zona pellucida and/or plasma membrane (Gwatkin et al. 1973; Gulyas & Schmell, 1980). Trypsin- or peroxidase-like enzymes have been proposed as being such agents. Fertilization-associated alteration of the properties of the oocyte surface, such as Con A agglutinability (Pienkowski, 1974) and alkaline phosphatase activity (Vorbrodt et al. 1977), has provided evidence to suggest that modifications of the molecular structure of the plasma membrane occur. It is widely assumed that some or all of the alterations may be the direct result of CG exocytosis (Schuel, 1978). Although this view is generally accepted for lower animals, experimental evidence to support this hypothesis is still unavailable for mammals. No suitable method has been developed for detecting the release of CG material during...
oocyte activation although detection for CG is possible in unfertilized oocytes by light microscopy (Austin, 1956). The absence of an appropriate method is one of the main factors responsible for the lack of a satisfactory explanation for these fertilization-associated changes in mammalian oocytes.

Over the past few years, we have studied the role of surface components, particularly carbohydrates, during mammalian fertilization. To date, the results obtained have provided clear evidence that many of the cellular events at fertilization are regulated by specific surface carbohydrates which are modified during sperm–egg interaction (Ahuja, 1985; Lee & Ahuja, 1987; Fraser & Ahuja, 1988). The uniallelic distribution of some of the carbohydrates on the interacting gametes provides the opportunity to develop a quantitative or semiquantitative method of monitoring sperm–egg interaction. In this paper, we report a simple staining method to observe the appearance and distribution of glycoconjugates in the PVS of mouse oocytes following activation. This phenomenon has been studied with lectins conjugated to fluorescein isothiocyanate (FITC). The results show that after oocyte activation, specific fucosyl- and sialyl-rich glycoconjugates are released into the PVS in a time-dependent manner similar to the exocytosis of CG.

Materials and methods

Source of gametes and embryos

Spermatozoa were obtained from (C57BL/6 Lac × CBA/Ca Lac)F1 hybrid males of proven fertility. Unfertilized oocytes were collected from (C57BL/6 Lac × CBA/Ca Lac)F1 hybrid or MF1 randombred females previously superovulated with intraperitoneal injections of 7.5 i.u. pregnant mares' serum gonadotrophin (PMSG, Folligon, Intervet, Cambridge, UK) followed 48–54 h later with human chorionic gonadotrophin (hCG, Chorulon, Intervet).

Fertilized oocytes and embryos were collected from similarly treated females that were placed with F1 hybrid males immediately after the injection of hCG and subsequently were found to have mated successfully by the presence of a vaginal copulatory plug.

Preparation of spermatozoa for fertilization in vitro

Spermatozoa were released from the cauda epididymis into modified Tyrode's T6 culture medium (Quinn et al. 1982) containing 15 mg ml⁻¹ bovine serum albumin (BSA fraction V, Sigma Chemical Co., Poole, UK). After incubation for 15 min at 37°C in an atmosphere of 5% CO₂ in air, cell debris and large aggregations of spermatozoa were removed from the suspension with a fine mouth-operated pipette. The sperm concentration was adjusted to 1–2×10⁶ spermatozoa ml⁻¹. The sperm suspension was overlaid with paraffin oil and incubated for a further 2 h for capacitation to take place. At this stage, oocytes with or without the zona pellucida were added to the sperm suspension to obtain fertilization in vitro.

Preparation of oocytes and embryos

(A) Unfertilized oocytes

Oocytes were released from the oviduct into M2 medium (Fulton & Whittingham, 1978) containing 4 mg ml⁻¹ crystalline BSA (Miles Scientific, Slough, UK) 14–15 h after the injection of hCG. The surrounding cumulus cells were dispersed with hyaluronidase (150 i.u. ml⁻¹, Bovine testes, Sigma) and the oocytes washed three times in M2 medium. Zona-free oocytes were prepared by a brief exposure to acid Tyrode's solution (pH 2.5) containing 4 mg ml⁻¹ polyvinylpyrrolidone (Sigma) followed by three washes in M2 medium. At this stage, the oocytes were either labelled immediately with the FITC-lectins as described below or labelled after artificial activation with the calcium ionophore A23187 (Calbiochem-Boehringer Corp., La Jolla, CA, USA) and ethanol and after fertilization in vitro.

For parthenogenetic activation, cumulus-free oocytes were washed in calcium-free PB1 medium (Whittingham, 1984) containing 4 mg ml⁻¹ crystalline BSA before incubating in fresh calcium-free PB1 medium containing 4 μM calcium ionophore A23187 previously dissolved in dimethylsulphoxide (DMSO, BDH Chemicals Ltd., Poole, UK) for 5–10 min. Extreme care was taken in the preparation of the calcium ionophore. All aliquots were made in a darkroom and protected from direct light during handling. In experiments involving A23187, the final concentration of DMSO was 0.01%. The oocytes were washed in phosphate-buffered saline (PBS, pH 7.4) containing 4 mg ml⁻¹ crystalline BSA (PBS+BSA) before labelling with FITC-lectins. For comparison, oocytes were also activated with 7% ethanol according to the method of Cuthbertson (1982).

For fertilization in vitro zona-intact or zona-free oocytes were added directly to the culture drop containing capacitated spermatozoa. Oocytes were removed from the culture drops at various time intervals, washed free of attached spermatozoa with M2 medium or fixed immediately to stop further sperm–oocyte interaction before assessing fertilization (Fraser, 1983) or labelling with FITC-lectins.

(B) Fertilized oocytes

Oocytes fertilized in vivo were obtained from mated females 16–18 h after the injection of hCG. Cumulus cells were removed as described above and oocytes showing the presence of a sperm tail or swelling of the sperm head within the oocyte cytoplasm were selected for labelling with FITC-lectins.

(C) Embryos

Embryos were either obtained from mated animals at various times after hCG injection or after in vitro fertilization and culture to the appropriate stage of preimplantation development.

Labelling of oocytes and embryos with FITC-lectins

Three different lectins, LPA (specific for sialic acid) from Limulus polyphemus, FBP (specific for fucose) from Lotus
tetragonolobus and UEA I (specific for fucose) from *Ulex europaeus* (E-Y Labs, San Mateo, CA, USA) were chosen to label the oocytes and embryos. Aliquots of the lectin stock solutions were stored at −20°C until use except LPA which was stored at 5°C. They were thawed and diluted with PBS + BSA to give a final concentration of 200 µg lectin ml⁻¹. After centrifugation the clear supernatant was used for labelling. Oocytes were either labelled fresh (unfixed) or after fixation with 3% formalin in PBS for 10 min. After incubation with lectins for 30 min at room temperature, they were washed thoroughly in PBS + BSA for 15 min to remove the unbound FITC-lectins. Control oocytes were incubated in the presence of glycoconjugates known to be specific for the lectins. Fucoidan (Sigma) at 5 mg ml⁻¹ was used for experiments with FBP and UEA I and fetuin IV (Sigma) at 4 mg ml⁻¹ for experiments with LPA.

**Fluorescence microscopy**

Labelled oocytes were mounted between a coverslip and a glass slide supported by four columns of a mixture of vaseline and wax (20:1). The drop containing the oocytes or embryo was sealed with paraffin oil. The slide was observed under an Ortholux Leitz epifluorescence microscope and the u.v. light was provided by a mercury lamp. Observations were made with ×25 and ×50 objectives together with a No. 3 blue filter. The photographs were taken by a Vario-Orthomat on HP5 Ilford film.

**Results**

**Lectin-binding properties of mouse oocytes**

The plasma membrane and the zona pellucida of freshly ovulated unfertilized oocytes showed no binding with the three different FITC-lectins, LPA, FBP and UEA I and FBP (Table 1). In some oocytes, the first polar body was brightly labelled nonspecifically throughout the cytoplasm. In contrast, the plasma membrane of oocytes fertilized in vivo was labelled brighty. The labelling pattern on fertilized oocytes appeared as a complete or partial ring surrounding the plasma membrane but this depended upon the orientation of the oocyte (Fig. 1A,B). No binding was apparent on the zona pellucida or on the surface of the second polar body. A careful examination of the oocyte after exposure to any one of the three lectins suggested that the fluorescence was due to lectins binding exclusively to the surface of the plasma membrane. To eliminate the possibility of antigen transfer from spermatozoa (Gaunt, 1983; Jones *et al.* 1985) oocytes were activated with either the calcium ionophore A23187 or ethanol. All treated oocytes showed a positive labelling (Fig. 1C,D). Labelling was completely inhibited in the presence of appropriate saccharides (data not shown) and no differences in the pattern and intensity of labelling could be detected between fertilized and activated oocytes (Fig. 1).

**Distribution of labelled glycoconjugates on the oocyte surface**

When the fluorescent labelling pattern was closely examined through different focal planes at higher magnification (×50 objective), a punctate distribution of labelled glycoconjugates was seen on the surface of the oocyte plasma membrane (Fig. 2). Removal of the zona pellucida either before or after labelling did not interfere with the appearance or distribution of the punctate fluorescent pattern, indicating its exclusive confinement to the oocyte surface. Also the same labelling pattern was present on fixed and unfixed oocytes thereby ruling out the possibility of phagocytic capture of the FITC-lectins.

**Time course of appearance of glycoconjugates after oocyte activation**

To determine whether the appearance of the fluorescent material on the oocyte surface was associated with CG release, zona-intact and zona-free oocytes were examined at various times after fertilization in *vitro*. Zona-intact oocytes began to show lectin labelling at about 25 min and reached a maximum between 50 and 60 min after addition of the oocytes to capacitated spermatozoa (Table 2). Lectin binding on the surface of zona-free oocytes appeared at approximately 15 min and reached a maximum 25 min after addition to capacitated spermatozoa. The progressive increase

<table>
<thead>
<tr>
<th>Lectins</th>
<th>Unfertilized oocytes</th>
<th>Fertilized oocytes†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pb1</td>
<td>ZP</td>
</tr>
<tr>
<td>LPA (<em>Limulus polyphemus</em>)</td>
<td>−/+*</td>
<td>−</td>
</tr>
<tr>
<td>FBP (<em>Lotus tetragonolobus</em>)</td>
<td>−/+</td>
<td>−</td>
</tr>
<tr>
<td>UEA I (<em>Ulex europaeus</em>)</td>
<td>−/+</td>
<td>−</td>
</tr>
</tbody>
</table>

−, negative; *, positive labelling.

Pb1, 1st polar body, Pb2, 2nd polar body; ZP, zona pellucida; PM, plasma membrane.

*The first polar body of unfertilized oocytes was labelled occasionally.
† Labelling pattern similar after artificial activation with ethanol or the calcium ionophore A23187.
in the number of oocytes showing intense labelling was found to be directly related to the fertilization rate and the known time course of CG release (Sato, 1979; Fraser, 1983). Fertilization rates for zona-intact and zona-free oocytes at the final sampling time were 96.0 and 92.0%, respectively (see Table 2 for the final sampling time). The labelling intensity was affected neither by the presence of the zona pellucida (Fig. 3F,H) nor by the number of spermatozoa fusing with the zona-free oocytes. Identical results were obtained with LPA and FBP (data not shown). After activation with A23187, the number of labelled oocytes reached a maximum within 5 s (Table 2). The calcium ionophore A23187 is well known for causing instantaneous release of CG in many species (Steinhardt et al. 1974). The distribution of the glycoconjugates and the number of fluorescing spots per unit area of oocyte surface were similar in fertilized and artificially activated oocytes.

**Fate of glycoconjugates during preimplantation**

The fate of glycoconjugates in oocytes fertilized *in vitro* was followed during culture. Shortly after the expected time of CG exocytosis, the even and discrete distribution of the lectin-labelled material changed and it appeared to coalesce to form larger aggregates over oocyte surface by the pronucleate stage i.e. at about 6 to 7 h postfertilization (Fig. 4C–F). Eventually, a large clump of the lectin-labelled material formed within the cleavage furrow of the early 2-cell embryo i.e. at about 22 h postfertilization (Fig. 4G,H). The glycoconjugates were not detected by the late 2-cell stage at about 36 h postfertilization and further labelling during subsequent stages of preimplantation development i.e. 4-cell–blastocyst stage was also negative. Preimplantation embryos obtained directly from the reproductive tract showed a similar pattern of labelling.
Discussion

Our present observations indicate that the glycoconjugates identified on the oocyte surface by the binding of LPA, UEA I and FBP originated from within the oocyte since they were not present on the surface of the unfertilized oocyte. Furthermore, they appeared in the PVS and on the oocyte plasma membrane shortly after sperm penetration and artificial activation. The lectin binding did not result from the phagocytotic activity of plasma membrane since fixed oocytes showed a similar intensity of lectin binding.
Third, the release of the glycoconjugates occurred in a 600 5.

Spermatozoa

ity and the sizes of the labelled spots was seen with hamster oocytes (Austin, 1956). While the initial

the known rate of CG discharge and sperm penetration during fertilization in the mouse (Sato, 1979; Fraser, 1983). A gradual increase in labelling intensity was due to the adhesion of more CG material on the oocyte surface (Fig. 3F,H), the enhancement noted at the later stages (Fig. 4B,D) appeared to be due to the coalescence of smaller particles to form larger aggregates.

Previous studies involving various enzyme inhibitors have suggested indirectly that trypsin (Gwatkin et al. 1973; Barros & Yanagimachi, 1971) or peroxidase-like enzymes (Gulyas & Schmell, 1980) enclosed within the CG membranes are released during oocyte activation. They disperse rapidly through the PVS modifying zona proteins (Florman & Wassarman, 1985) which prevent the further passage of spermatozoa through the zona pellucida. Whether these enzymes influence the properties of the oocyte plasma membrane is unknown and the present data do not modify these concepts significantly.

The most striking observation of this study is the appearance of fucosyl- and sialyl-glycoconjugates in the PVS shortly after oocyte activation. In view of the observed timing of glycoconjugates release (Table 2) correlating so well with the known timing of CG exocytosis and sperm penetration (Sato, 1979; Fraser, 1983), it is tempting to speculate that the glycoconjugates somehow play a crucial role in initiating the block to polyspermy at the level of the zona pellucida as well as the plasma membrane. The fucosylated and sialylated components might initiate the ‘zona reaction’ (Austin, 1961) by interacting with zona proteins, particularly the ZP3, a 83×10^3 M_r protein, known for its sperm receptor properties (Bleil et al. 1981). Alternatively, the glycoconjugates may trigger a highly effective ‘oolemma’ block to polyspermy by modifying the plasma membrane in a manner analogous to the sea urchin eggs where the cortical saccharides form a hyalin layer (Citkowitz, 1971; Kane, 1973; McBlaire & Carroll, 1980). The establishment of a ‘fast block’ to polyspermy by influencing the electrical properties of the plasma membrane (Jaffe & Cross, 1986) and/or a ‘slow block’ by mechanochemical neutralization of sperm binding sites on the oocyte membrane (Ahuja, 1985) may both involve interaction with glycoconjugates.

A further observation in this study was the coalescence of the glycoconjugate material on the oocyte surface membrane until it eventually formed a single large clump in the cleavage furrow of the majority of early 2-cell embryos i.e. at about 22 h postfertilization. There was no evidence for the presence of this material during the remainder of preimplantation development. At present, there is no explanation for this phenomenon. However, it is plausible to consider that CG exocytosis is coupled with endocytosis of membrane by the penetrated oocyte (Donovan & Hart, 1982, 1986; Fisher & Rebhun, 1983; Carron & Longo, 1983). In mouse embryos, endocytotic activity

<table>
<thead>
<tr>
<th>Mode of treatment</th>
<th>Time after treatment (min)</th>
<th>% oocytes (no. oocytes) with FITC–UEA I fluorescence when zona intact</th>
<th>% oocytes (no. oocytes) with FITC–UEA I fluorescence when zona removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spermatozoa (1–2×10^6 ml^-1)</td>
<td>10</td>
<td>0 (34)</td>
<td>0 (34)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0 (38)</td>
<td>75.4 (57)</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.3 (30)</td>
<td>93.3 (43)</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>19.0 (63)</td>
<td>93.8 (48)</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>40.0 (50)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>76.1 (71)</td>
<td>–</td>
</tr>
<tr>
<td>A23187 (4 μμ)</td>
<td>1/12 (5 sec)</td>
<td>100 (35)</td>
<td>–</td>
</tr>
<tr>
<td>Medium only (Control)</td>
<td>60</td>
<td>0 (30)</td>
<td>0 (24)</td>
</tr>
<tr>
<td>DMSO (0.01 %) (Control)</td>
<td>10</td>
<td>0 (32)</td>
<td>–</td>
</tr>
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</table>

The bright labelling of the first, but not the second, polar body in some oocytes is thought to be non-specific, resulting probably from the exposure of CG material during its disintegration or perhaps from the accumulation of unbound fluorescein as experienced under conventional light microscopy as experienced with hamster oocytes (Austin, 1956). While the initial
Fig. 3. The distribution of labelled glycoconjugates on surface of zona-free oocytes sampled at various times after exposure to capacitated spermatozoa. Samples of oocytes were labelled with FITC-UEA I at 0 (A,B), 10 (C,D), 15 (E,F), and 30 min (G,H) after the addition of spermatozoa. Note the absence of labelling in the oocyte incubated without spermatozoa (A,B) or during the early stages of incubation (C,D). Consecutive stages of fusion with spermatozoa (arrows) are shown in E–H. The glycoconjugates visualized during the later stage (G,H) appear to be of greater size than the spots of earlier stages (E,F). Bar, 20 μm. Objective ×50.
Fig. 4. The fate of glycoconjugates after fertilization. Zona-intact oocytes were fertilized in vitro before being labelled with FITC-UEA I. Approximately 45 min after incubation with capacitated spermatozoa the release of the glycoconjugates was complete as judged by UEA I-binding pattern (A,B). Subsequent culture of in vitro fertilized oocytes in M16 medium resulted in the aggregation of glycoconjugates into larger clumps (C–F). This process continued up to the first cleavage division when a large single aggregate was located in the cleavage furrow (G,H). This single aggregate disappeared completely by the late 2-cell stage. Bar, 20 μm. Objective ×50.
has been demonstrated at the time of compaction at the 8-cell stage (Fleming & Pickering, 1985) and it may be responsible for the disappearance of the labelled glycoconjugates by the late 2-cell stage as observed in this study.

This is the first demonstration of the release of fucosyl- and sialyl-glycoconjugates in association with the cortical reaction in fertilized and artificially activated mouse oocytes. The physiological role of the glycoconjugates has yet to be defined. However, it is significant that the stimulation of exocytosis in certain other cell systems is associated with the release of factors, predominantly proteoglycans and glyco-aminoglycans, which exert a profound influence on the subsequent growth and development of cells (Rapraeger et al. 1986). In certain vertebrates and invertebrates, CG material has been shown to support early development by establishing an adhesive interaction among blastomeres (Citkowitz, 1971; Kane, 1973). When combined with appropriate molecular and ultrastructural approaches the relatively simple detection system described here should help to quantify the kinetics of exocytosis of CG and to determine the role of cortical glycoconjugates in the block to polyspermy and in the development of the early mammalian embryo.

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References


S. H. Lee and others


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