Immunogold study on lectin binding in the porcine zona pellucida and granulosa cells

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An ultrastructural localization of lectin receptors on the zona pellucida (ZP) of porcine antral oocytes and on the granulosa cells was performed using a panel of horseradish peroxidase-labelled lectins in conjunction with antiperoxidase antibody and protein A-gold. In some cases, lectin incubation was preceded by sialidase digestion. WGA-, Con-A-, UEA-I-, RCA-I-, PNA- and SBA-reactive sites were distributed differently in the porcine ZP. Sialidase digestion increased the positivity obtained with RCA-I and it was necessary to promote PNA and SBA reactivity. These results indicated that the ZP contained N-acetylglucosamine, α-mannose, α-fucose, β-Gal-(1-4)GlcNAc, β-Gal-(1-3)GalNAc, β-GalNAc and sialic acid residues. We also observed the presence of vesicles in both the ooplasm and granulosa cells, showing a similar lectin binding pattern to that of the ZP, thus suggesting that the oocyte and granulosa cells are the site of synthesis of ZP glucidic determinants.

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Key words: Zona pellucida, granulosa cells, lectin.

Paper accepted on April 16, 2003

European Journal of Histochemistry
2003; vol. 47 issue 4 [Oct-Dec]: 353-358

The Zona Pellucida (ZP), a unique extracellular coat that surrounds the mammalian oocytes and preimplantation embryo, plays essential functions during the fertilisation and early stages of zygote development (Wassermann, 1994; Sinowatz et al., 2001). The ZP of most mammals is composed of three major, highly glycosylated glycoproteins, which, in the mouse, are called ZPA, ZPB and ZPC (Bleil and Wassermann, 1990). Zona carbohydrates strongly influence the physicochemical properties of ZP glycoproteins and participate in sperm-zygote recognition events, probably as ligands for complementary carbohydrate-binding proteins on the sperm surface.

Comparative histochemical studies have shown species-dependent variations in the expression and distribution of carbohydrates throughout the ZP (Parillo et al., 1996, 1998, 1999, 2000, 2001a,b; Verini Supplizi et al., 1996; Chapman et al., 2000). The organisation of the ZP in morphological distinct bands, which have been demonstrated by light microscopic studies in the above mentioned investigations, was also found in the rat, mouse and hamster at the ultrastructural level (Roux and Kan, 1991; Avilés et al., 1996, 2000; El Mestrah and Kan, 2001).

The site of ZP glycoprotein production may vary among species. In the majority of mammals, both the oocyte and granulosa cells are involved in the origin of the ZP (Sinowatz et al., 2001). Conversely, the oocyte is the only source of the ZP in mice (Bleil and Wassermann, 1980; Fléchon et al., 1984; Epifano et al., 1995) and rats (Avilés et al., 1994). The involvement of both the oocyte and follicular cells in the secretion of the porcine ZP was demonstrated in an immunocytochemical study carried out using antibodies directed against porcine ZP3a and ZP3b glycoproteins (Sinowatz et al., 1995). Finally, in this regard, it has been demonstrated that in the hamster, besides oocyte and/or granulosa cells, involved in ZP production, the oviductal cells con-
tribute to the synthesis of certain ZP glycoproteins after oocyte ovulation (Kan et al., 1989; Roux and Kan, 1991; El-Mestrah and Kan, 2001).

In the present cytochemical study, we used hors eradish-peroxidase-conjugated lectins to analyse the ultrastructural distribution of the glycoprotein glucidic residues present in the porcine ZP, in the ooplasm and in the granulosa cells.

Materials and Methods

Oocytes-granulosa cell collection
Porcine oocyte-granulosa cell complexes were aspirated from antral follicles (3-4 mm diameter) present in the ovaries of adult sows immediately after slaughter.

Cytochemical labelling
The oocyte-granulosa cell complexes were fixed in 2.5% gluteraldehyde in 0.1 M cacodylate buffer pH 7.4 for 2hr at room temperature. Subsequently, the oocyte-granulosa cell complexes were processed for electron microscopy according to the procedures previously described by Menghi et al. (1996a,b) and Scala et al. (1992) which were partially modified. The cumulus masses were dehydrated in a series of 50%, 70% and 90% acetone and embedded in Bioacryl resin (Bio-optica, Milan, Italy). Thereafter, ultrathin sections (about 80 nm thick), which were placed on parlodion-coated 200 mesh nickel grids, were obtained from these samples. The nickel grids were preincubated at room temp. for 10 min in 0.5M TBS (Tris Buffered Saline) pH 7.4 containing 0.25% BSA (Bovine Serum Albumine) and then, incubated at room temp. for 3h with horseradish-peroxidase (HRP) conjugated lectins diluted in TBS, pH 7.4 plus 1% BSA. The lectins used, their carbohydrate specificity and the optimal concentrations are shown in Table 1. After lectin incubation, sections were rinsed with 0.05M TBS containing 1% BSA and reacted with anti-HRP antibody (raised in rabbit and diluted 1:100 in TBS-1% BSA) at room temp. for 1h. Then, sections were washed with 0.05M TBS-1% BSA and treated with protein A gold (10 or 20 nm) diluted 1:100 in TBS-1% BSA at room temp. for 1h. Finally, after counterstaining with uranyl acetate and lead citrate, sections were examined with a Philips EM 208 electron microscopy at 80 KV.

All the HRP-labelled lectins, the anti-HRP antibody and protein A gold were purchased from Sigma Chemical Co (St. Louis, MO, USA).

Enzymatic treatment
Before WGA, PNA, SBA, RCA-I and DBA lectin incubation, tissue sections were digested with sialidase (neuraminidase type V, from Clostridium perfringens) at a concentration of 0.86 U/mL in acetate buffer pH 5.5 containing 10 mM CaCl₂ for 16h at 37° C (Menghi et al., 1996b).

Controls
The control for the specificity of lectin cytochemistry was carried out either by adding the specific competing sugar at a concentration of 0.2-0.4 M in the lectin solutions or by omitting the anti-peroxidase antibody or lectin HRP conjugates. Controls for enzymatic digestion were performed by substituting the enzyme with its corresponding buffer.

Results
Zona pellucida
The ZP of oocytes exhibited heavy to moderate labelling over its entire thickness when labelled with WGA and Con-A (Figures 1a-2a). Sialidase digestion did not modify WGA positivity. UEA-I lectin showed a slight reactivity preferen-
tially located to the inner region of the ZP (Figure 3).

Oocytes incubated with RCA-I showed a differential labelling throughout the ZP. A high concentration of gold particles was preferentially associated with the inner layer of the ZP, whereas they progressively decreased towards the portion in contact with the follicular cells (outer ZP) (Figure 4).

Sialidase digestion prior to RCA-I staining promoted new binding sites over the outer portion of the ZP (Figure 5a).

SBA, after enzymatic degradation, preferentially labelled two bands of the ZP: the inner and intermediate bands. The outer part of the ZP showed scarce binding sites (Figure 6).
PNA reactive sites were slightly and sparsely distributed in the ZP only after the cleavage of sialic acid residues. No labelling was observed with DBA and LTA.

**Ooplasm**

A population of vesicles reactive to WGA (Figure 1b), Con-A (Figure 2b) UEA-I, sialidase/RCA-I (Figure 5b), sialidase/PNA and sialidase/SBA was evidenced in the ooplasm. Other cellular organelles, such as mitochondria and endoplasmic reticulum, were devoid of any labelling.

**Granulosa cells**

The cytoplasm of the granulosa cells contained numerous vesicles that displayed reactivity with WGA (Figure 1c), Con-A (Figure 2c), UEA-I, sialidase/RCA-I (Figure 5c), sialidase/PNA and sialidase/SBA.

**Controls**

Control sections did not show any appreciable reactivity, confirming the specificity of lectin labelling.
Discussion

The porcine ZP is composed of three structurally and immunologically distinct glycoproteins denominated PZP1, PZP3α and PZP3β (Dunbar et al., 1980; Hedrick and Wardrip, 1986; Yurewicz et al., 1991). PZP3α and PZP3β are highly glycosylated and possess both N- and O-linked oligosaccharide chains (Yonezawa et al., 1997; Yurewicz et al., 1992). It has been suggested that carbohydrate chains of a mixture of PZP3α/PZP3β have marked functional relevance in sperm binding. In the present study, the ultrastructural identification of the porcine ZP glycoproteins, allowed us to obtain a detailed in situ localization of the oligosaccharide chains. The reactivity of the ZP to WGA and Con-A indicates the occurrence of GlcNAc and α-mannose residues, respectively. These carbohydrates are uniformly distributed throughout the entire thickness of the ZP but GlcNAc residues are more abundant than α-mannose ones. UEA-I reactivity suggests the presence of α-fucose residues which are preferentially located in the inner region of the ZP. In our previous histochemical study carried out in the porcine ZP with a light microscope, Con-A and UEA-I-binding sites were not identified (Parillo et al., 1996). This disagreement between the results obtained at light and electron microscopy could probably be explained by the scarce amounts of α-mannose and α-fucose residues. Fucose was identified by UEA-I, but not by LTA, which has a similar nominal specificity for α-fucose as UEA-I does. This apparent discrepancy in lectin behaviour could be due to the different types of linkages that binds fucose to penultimate sugars. In particular, fucose visualized by LTA seems to be involved in the linkages with GlcNAc and/or D-Gal, while fucose recognized by UEA-I seems to be bound to D-Gal (Menghi et al., 1989).

The positivity of SBA and PNA lectins only after sialidase digestion indicates the presence of sialic acid-GalNAc and sialic acid-β-Gal-(1-3)GalNAc as terminal sequences in sialoglycoproteins. These sugar sequences were differently distributed throughout the zona matrix. In fact, the disaccharide sialic acid-GalNAc was present, above all, in the inner and intermediate bands of the ZP where-as the trisaccharide, sialic acid-β-Gal-(1-3)GalNAc, was sparsely distributed through the ZP. RCA-I reactivity indicates the presence of the terminal disaccharide, βGal-1-4GlcNAc, which was located more densely in the area closest to the oocyte plasma membrane. This dimer was also found penultimate to sialic acid and distributed in the outer and intermediate portions of the ZP.

Our findings are in agreement with previous investigations carried out on several mammalian ZPs. In fact, using lectin histochemistry at light and electron microscopy, it has been demonstrated that, in the ZP, an inner and outer layer can be identified, suggesting that the carbohydrate moieties of the ZP are asymetrically distributed (Avilés et al., 1994, 2001; Parillo et al., 1996, 1998, 1999, 2001a,b; Verini Supplizi et al., 1996; Roux and Kan, 1991; El Mestrah and Kan, 2001).

Indeed, the ultrastructural localization allowed us to identify three distinct zones containing different lectin receptors in the porcine ZP: the area localised close to the oocyte (inner ZP), an intermediate region and a portion contacting the granulosa cells (outer ZP). Glycoproteins containing terminal βGal-1-4GlcNAc and α-fucose are confined prevalently in the inner ZP, those with terminal sialic acid-GalNAc are located mainly in the intermediate band while sialoglycoproteins with terminal sialic acid-βGal are more abundant in the outer layer.

Sialic acid, detected histochemically (Parillo et al, 1996) and biochemically (Dunbar et al., 1980) in the ZP of the pig, contributes to the high heterogeneity of the carbohydrate portion of the ZP matrix. It has been suggested that this terminal sugar keeps its associated glycoproteins impotent until the sperm-egg interaction has taken place.

Specialized vesicular aggregates, showing a lectin

![Figure 6. Sialidase/SBA staining. (a). SBA bound preferentially the inner and intermediate bands of the ZP after the cleavage of sialic acid residues. (b). x 17000. O, ooplasm; PS, perivitelline space; FC, follicular cell.](image-url)
binding pattern superimposable to that of the ZP, were observed in the ooplasm as described in some laboratory rodents, suggesting their involvement as intermediary vehicles in the final processing and secretion of glycosylated ZP proteins (Avilés et al., 1994, 2000; El Mestrah and Kan, 2001). In addition, the detection of numerous vesicles in the cytoplasm of granulosa cells, with a pattern of lectin labelling resembling that of the ZP, suggests that also these cells could be involved in the active synthesis and secretion of the ZP glycoproteic material.

In conclusion, the present investigation provides an accurate account of the strategic localisation of lectin receptors throughout the porcine ZP, indicating that glycoproteins with O-linked oligosaccharides prevail in the inner and intermediate portions of the ZP, whereas glycoproteins with N-linked oligosaccharides are mainly localised in the outer ZP. In addition, this work enabled us to discover some glucidic residues, such as mannose and fucose, that were not revealed at the light microscope.

Acknowledgments

The authors wish to express their gratitude to Prof. G. Menghi (University of Camerino, Italy) for her helpful suggestions and criticism. The excellent technical assistance of Ms. M.G. Mancini is also gratefully acknowledged.

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