Distribution of carbohydrates in the zona pellucida of human oocytes

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The purpose of the present study was to investigate the distribution pattern of carbohydrates in the zona pellucida of human oocytes using lectins and ruthenium red as histochemical probes. For lectin analyses, oocytes that failed to undergo fertilization following *in vitro* insemination were collected, washed, fixed with glutaraldehyde and embedded in araldite. For ruthenium red labelling, the oocytes were fixed with glutaraldehyde containing ruthenium red, post-fixed with OsO₄ and embedded in araldite. Araldite sections (1 µm) were de-resined with sodium methoxide, rehydrated, labelled with ten different biotinylated lectins as probes and avidin–biotin–peroxidase complex as visualant, and examined under a light microscope. The zonae pellucidae of all oocytes studied exhibited a common lectin-binding pattern, expressed in intense binding of lectins from *Concanavalia ensiformis* (ConA), *Lens culinaris* (LCA), *Ricinus communis* (RCA-I), wheat germ agglutinin (WGA), and of succinylated WGA (S-WGA). Peanut lectin (PNA) bound to the zona pellucida only after neuraminidase treatment, whereas the lectins from *Griffonia simplicifolia* (GS-I), *Dolichos biflorus* (DBA), *Ulex europaeus* (UEA-I) and soybean (SBA) did not bind at all. There was almost no binding of ruthenium red to the matrix of the zona pellucida. The results indicate that the human zona pellucida is characterized by normally exposed mannosyl, N-acetylglucosaminyl and β-galactosyl residues. In addition, it contains masked βGal-(1–3)GalNAc sugar sequences that can be exposed only after removing terminal sialic acid residues. The presence of sialic acid in the human zona pellucida, which is not expressed as an increase in the binding capacity of the polycationic probe (ruthenium red), has not been reported in any of the mammalian zonae pellucidae previously studied.

Introduction

The mammalian oocyte is surrounded by a transparent, extracellular coat called the zona pellucida, which plays an important role during fertilization. It restricts interspecies fertilization, induces the acrosome reaction in bound spermatozoa, and assists in establishing the zona block to polyspermy after sperm penetration (Yanagimachi, 1988; Saling, 1989).

In a wide variety of species studied, the zona pellucida is composed of only a few glycoproteins. It has been suggested that carbohydrate determinants of the zona pellucida glycoproteins are involved in the sperm–egg interaction (Macek and Shur, 1988; Miller and Ax, 1990; Wassarman, 1990). The most studied mammal in this respect is the mouse, in which the zona pellucida has been shown to consist of three glycoproteins, ZP1, ZP2 and ZP3, each of which presents a unique polypeptide chain and oligosaccharides (Wassarman, 1988). It has been demonstrated that the sperm–egg interaction in the mouse involves specific sugar residues such as α-galactose (Gal) of O-linked oligosaccharides of ZP3 (Bleil and Wassarman,

1988) or N-acetylglucosaminyl (GlcNAc) residues (Miller *et al.*, 1993) serving as sperm-binding sites, and that it also involves the polypeptide chain as an inducer for the acrosome reaction (Florman *et al.*, 1984; Florman and Wassarman, 1987).

Skutelsky *et al.* (1994) demonstrated that the distribution of zona pellucida carbohydrates from oocytes of different mammals were different, suggesting that these differences might be important in the control of species-specific gamete interactions. Shabanowitz and O’Rand (1988) studied the composition of the human zona pellucida surrounding fertilized and unfertilized eggs. They reported that, under reducing conditions, zonae from unfertilized eggs can be separated into three acidic proteins, also designated ZP1, ZP2 and ZP3. It was later reported that ZP3 consists of two isomer chains (Shabanowitz, 1990); its amino acid sequence was also analysed (Chamberlin and Dean, 1990).

It may be assumed that, as in other mammals, the interaction between gametes in human reproduction might also depend on species-specific recognition by carbohydrates expressed on the zona pellucida surface, and on changes in the distribution pattern of these carbohydrates following maturation and fertilization. Thus, it is important to localize and characterize the spatial distribution and availability of specific sugar residues

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Table 1. Lectins used for identifying carbohydrate residues in zona pellucida of human oocytes

Origin of lectin	Common name	Acronym	Concentration ($\mu\text{g ml}^{-1}$)	Major sugar specification ^a	Binding inhibitor
<i>Arachis hypogea</i>	Peanut	PNA	20	$\beta\text{Gal}-(1-3)\text{GalNAc}$	Lactose
<i>Griffonia simplicifolia</i>	Griffonia	GS-I	50	$\alpha\text{-D-Gal}$	Lactose
<i>Concanavalia ensiformis</i>	Jack bean	ConA	10	$\alpha\text{D-Glc}$, $\alpha\text{-D-Man}$	$\alpha\text{-D-methyl-Man}$
<i>Dolichos biflorus</i>	Horse gram	DBA	10	$\alpha\text{-D-GalNAc}$	$\alpha\text{-D-GalNAc}$
<i>Glycine max</i>	Soybean	SBA	10	$\alpha\text{-D-GalNAc}$, $\alpha\text{-D-Gal}$	$\alpha\text{-D-GalNAc}$
<i>Lens culinaris</i>	Common lentil	LCA	10	$\alpha\text{-D-Glc}$, $\alpha\text{-D-Man}$	$\alpha\text{-D-methyl-Man}$
<i>Ricinus communis</i>	Castor bean	RCA-I	50	$\beta\text{-D-Gal}$	Lactose
<i>Triticum vulgaris</i>	Wheat germ	WGA	50	$[\beta-(1-4)\text{-D-GlcNAc}]_2\text{NeuNAc}$	NeuNAc
<i>Triticum vulgaris</i>	Succinyl-WGA	S-WGA	10	$[\beta-(1-4)\text{-D-GlcNAc}]_2$	$\beta\text{-D-GlcNAc}$
<i>Ulex europaeus</i>	Gorse	UEA-I	10	$\alpha\text{-L-fucose}$	$\alpha\text{-L-fucose}$

^aGal: galactose; GalNAc: N-acetylgalactosamine; GlcNAc: N-acetylglucosamine; Man: mannose; NeuNAc: N-acetylneuraminic acid (sialic acid).

in the human zona pellucida, and to detect possible changes in their distribution in correlation with developmental processes.

Lectins are sugar-binding proteins or glycoproteins of nonimmune origin that agglutinate cells or precipitate glycoconjugates having saccharides of appropriate complementarity (Goldstein *et al.*, 1980). In numerous studies, plant lectins have been used to inhibit sperm-egg adhesion (Oikawa *et al.*, 1973; Rosati *et al.*, 1978; Bolwell *et al.*, 1979; Ahuja and Bolwell, 1983; Barnum and Brown, 1983; Catt *et al.*, 1983; Del Pino and Cabada, 1987; Mori *et al.*, 1989). Lectins have been used to characterize the zona pellucida in hamsters, mice and pigs with light and electron microscopes (Nicolson *et al.*, 1975; Dunbar *et al.*, 1980; Wu *et al.*, 1984; Kan *et al.*, 1990). We analysed the distribution of lectin-binding patterns in various mammalian oocytes with both types of microscope (Shalgi *et al.*, 1991; Skutelsky, *et al.*, 1994). In the present study, we examined the distribution and availability of specific sugar residues in the zona pellucida of human oocytes. Since certain sugars, such as sialic acid or heparan sulfate, largely contribute to the charge properties of the oocyte membrane and extracellular matrix (Skutelsky *et al.*, 1992), we also used the polycationic histochemical probe ruthenium red (Luft, 1971), which is known to interact with sialic acid residues, to characterize the polyanionic constituents on the zona pellucida and associated structures.

Materials and Methods

In vitro fertilization protocol

Women ($n = 15$) scheduled for *in vitro* fertilization underwent controlled hyperstimulation with a GnRH analogue (either D-Trp⁶ (Decapeptyl; Ferring AG, Kiel) or buserelin acetate (Superfect; Hoechst AG, Frankfurt on Main)), and human menopausal gonadotrophin (hMG) (Pergonal, Teva Ltd, Kfar Sava), as reported by Ben-Nun *et al.* (1990). The oocytes were inseminated 4–6 h after oocyte aspiration with at least 10^{-5} motile spermatozoa obtained from semen samples by a swim-up procedure. Ham's F-10 solution (Irvine

Scientific, Irvine, CA), supplemented with 10% patient serum and immediately centrifuged at 1000 *g* for 2 min and heat inactivated was used as a culture medium. The oocytes were dissected out of the surrounding cumulus mass 16–18 h after insemination and examined for the presence of pronuclei. Those oocytes lacking pronuclei (categorized as 'not fertilized') were washed in PBS (pH 7.4) and fixed with 2.5% glutaraldehyde in 0.1 mol phosphate buffer l^{-1} (pH 7.4). All specimens were studied with the consent of the patients and represent material that would normally have been discarded.

Ruthenium red labelling

The oocytes were washed in PBS, fixed for 3 h with 2.5% glutaraldehyde containing 0.05% ruthenium red, washed with PBS, and post-fixed for 1 h with 1% OsO_4 in Veronal-acetate buffer (pH 7.4) containing 0.05% ruthenium red (Luft, 1971). The samples were then dehydrated in ethanol and propylene oxide and embedded in araldite.

Light microscope histochemistry

For araldite-embedded material, 1 μm thick sections were cut using a LKB ultramicrotome. The resin was removed by mounting the sections on microscope slides, treating with sodium methoxide, washing with methanol-benzene and acetone, and rehydrating by immersion in PBS. Endogenous peroxidase was blocked by incubation in 2% H_2O_2 at 37°C for 10 min. The sections were then covered with 1% BSA in PBS for 10 min, the excess was shaken off, and the slides around the tissue sections were blotted. They were then incubated with one of ten different biotin-labelled lectins (Vector Laboratories, Burlingame, CA; Table 1) for 30 min, washed three times in PBS and incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min, followed by three washes with PBS. The visualant, horseradish peroxidase, was activated by incubation for 8 min in PBS solution containing diaminobenzidine and H_2O_2 . After washing in tap water for 5 min, the sections were dehydrated and covered with a Merckoglass coverslip.

Fig. 1. Semithin araldite sections of human oocytes, stained with (a) concanavalin A (ConA), (b) common lentil lectin (LCA), (c) castor bean lectin (RCA-I), (d) wheat germ agglutinin (WGA), and (e) succinylated WGA. The zona pellucida of all the oocytes is intensely stained by the lectins. The binding of WGA is mainly confined to the outer and inner regions of the zona pellucida. Scale bar represents 100 μ m.

The lectins used in this study, their acronyms, concentrations, major sugar specificities, and corresponding sugars that inhibit their binding are given in Table 1. The concentrations selected were to reduce background binding to a minimum, but were rich enough to detect relatively low concentrations of specific sugar residues. Incubation of lectins with 0.2 mol of corresponding sugar haptens l^{-1} (Table 1) before and during the application of the lectin solution to the tissue sections served as specific controls. Incubation of avidin-biotin-peroxidase complex alone served as a nonspecific control. The lectin-stained slides were examined under a microscope and the staining was defined as positive or negative. The degree of staining intensity was appreciated semiquantitatively as weak, moderate or strong.

Electron microscopy

For evaluation of ruthenium red binding, ultrathin sections, 60 nm, of the araldite-embedded oocytes were prepared using a LKB-V ultramicrotome equipped with a diamond knife. The sections were stained with uranyl acetate and lead citrate and examined under a JEOL-100B electron microscope at 80 kV.

Results

Binding of lectins to the zona pellucida

The study was carried out on oocytes collected from 15 women, and included 32 oocytes that were singly embedded in araldite. The use of araldite as an embedding medium allowed the histochemical analyses to be performed on single oocytes, and the analyses to be performed on semithin ($< 1 \mu$ m thick) sections. This approach provided a considerably improved resolution of the histochemical reactions compared with paraffin wax sections.

Ten different lectins (Table 1) were examined for their capacity to bind to ovulated human oocytes. In all the oocytes studied, there was an intense binding of specific lectins to the zona pellucida, and the lectin-binding patterns of the different oocytes studied were very similar. The zona pellucida of all the oocytes displayed intense binding capacity for five of the lectins studied: Concanavalin A (ConA; Fig. 1a), the common lentil lectin LCA (Fig. 1b), the castor bean lectin RCA-I (Fig. 1c), wheat germ agglutinin (WGA; Fig. 1d) and succinyl-WGA (Fig. 1e).

The binding of ConA, LCA and RCA-I were generally evenly distributed throughout the entire thickness of the zona

Fig. 2. Araldite section of human oocytes stained with peanut lectin (PNA) (a) before or (b) after treatment of the sections with neuraminidase. Intense binding of PNA to the zona pellucida is seen only in the neuraminidase-treated oocytes. Scale bar represents 100 μ m.

pellucida (Fig. 1a, b, c), whereas that of WGA was considerably more intense at the inner and outer regions of the zona pellucida (Fig. 1d) leaving the central portion of the zona pellucida virtually unlabelled. Unlike WGA, the binding of succinyl-WGA was mainly restricted to the central part of the zona pellucida (Fig. 1e).

The lectins GS-I, SBA, DBA, UEA-I and PNA did not bind at all to the human oocytes (Fig. 2a). However, after treatment of the oocytes with neuraminidase, an intense binding of PNA was found to be evenly distributed throughout the zona pellucida (Fig. 2b).

Ruthenium red labelling

The binding of ruthenium red to the human oocyte was analysed in thin araldite sections using the transmission electron microscope. Deposition of ruthenium red was mainly observed as clustered material located in the extracellular matrix surrounding the oocytes (Fig. 3). A few dispersed clusters staining with ruthenium red also appeared within the perivitelline spaces. However, within the zona pellucida there was no noticeable binding of ruthenium red, except in occasional granules. There was also no ruthenium red labelling on the plasma membrane of the oocyte.

Discussion

The present study provides evidence that, as in other mammalian species studied, the zona pellucida of the human oocyte is characterized by a high concentration of saccharide residues capable of binding specific lectins.

By using a large selection of lectins as histochemical markers for zona pellucida carbohydrates, and semithin (< 1 μ m) araldite sections for histochemistry with the light microscope we were able to characterize both the chemical nature and the spatial distribution of the different sugar residues throughout the entire thickness of the zona pellucida.

We have recently shown (Skutelsky *et al.*, 1994) that the zonae pellucidae of different mammals are characterized by unique lectin-binding patterns, which probably represent species-specific variation in their carbohydrate distribution. The

lectin-binding pattern of the human zona pellucida has been found in this study to differ from that of other mammals studied (i.e. the human oocyte also displays a species-specific distribution pattern of carbohydrates).

The most pronounced differences between the human zona pellucida and other mammalian zonae pellucidae studied were in the binding of ConA, LCA, S-WGA and PNA. The binding of ConA and LCA, which was relatively high compared with that of other mammals (Skutelsky *et al.*, 1994), indicates the presence of a high concentration of D-mannose residues in the human zona pellucida, probably reflecting a high content of asparagine-linked oligosaccharides. On the basis of a sugar competition assay, Mori *et al.* (1989) have suggested that the presence of D-mannose residues on the zona pellucida is important in human fertilization.

LCA was previously shown to bind with high affinity to poly-N-acetyllactosamine-like glycopeptides, which contain an α -L-fucosyl residue attached to the asparagine-linked N-acetylglucosamine (GlcNAc) residue (Kornfeld *et al.*, 1981). The presence of high quantities of poly-N-acetyllactosamine was indeed demonstrated in sugar chains of porcine zonae pellucidae glycoproteins (Mori *et al.*, 1991; Yurevicz *et al.*, 1991). The significance of fucosyl residues in human fertilization has also been demonstrated (Huang *et al.*, 1982; Tam *et al.*, 1990), suggesting that L-fucose may be part of a recognition signal between spermatozoa and eggs. At fertilization, the spermatozoon interacts initially with the mature cumulus cells and the extracellular matrix surrounding them, and then binds to the zona pellucida. Tesarik and Kopečný (1986) reported that during the preovulation phase the human oocyte and the cumulus cells both incorporate glucosamine and fucose, which serve as precursors for proteoglycans that can later be found in the zona pellucida and the cumulus intercellular matrix.

The high reactivity of WGA, which was confined mainly to the outer and inner regions of the zona pellucida, may reflect a high content of either GlcNAc or sialic acid residues or both (Monsigny *et al.*, 1980) at these regions. In contrast, the binding of the succinylated derivative of WGA (S-WGA), which is known to bind only to GlcNAc and not to sialic acid residues (Monsigny *et al.*, 1980), was generally more intense and was evenly distributed throughout the entire thickness of the zona pellucida. The failure of WGA to bind to the inner portions of the zona pellucida, as opposed to S-WGA, has been previously described in the zona pellucida of rodents (but not in that of canine, feline and porcine oocytes); it was suggested that there are positively charged constituents that interfere with the binding of positively charged lectins, thus preventing them from binding to the inner regions of the zona pellucida (Skutelsky *et al.*, 1992).

The only lectin-binding reactivity that was completely unique to humans was with PNA. This lectin bound intensely to the human zona pellucida only after pretreatment of the oocytes with neuraminidase, a process that cleaves off the terminal sialyl residues, thus exposing normally masked penultimate sugar residues. The ability of neuraminidase to expose PNA-binding sites in the human zona pellucida expresses two unique features of this structure in humans: (1) the presence of cleavable sialyl residues serving as a major terminal sugar in the human zona pellucida; and (2) the existence of high amounts of PNA-binding sugar sequences (i.e. β Gal-(1-3)GalNAc; Lotan

Fig. 3. Electron micrograph of a human oocyte (O) labelled with ruthenium red during fixation. Ruthenium red labelling is seen on cellular fragments in the perivitelline space and in small particles in the extracellular matrix. Except for a few dispersed particles, the matrix of the zona pellucida (ZP) is virtually unlabelled (arrows). Scale bar represents 0.5 μ m.

et al., 1975) only as penultimate to terminal sialyl residues. The existence of PNA-binding sites has been observed in murine oocytes (Skutelsky *et al.*, 1994). However, in mice these sites appeared as normally exposed sugars and neuraminidase treatment did not expose such sites any further. The disaccharide β Gal-(1-3)GalNAc was also shown to appear as a major core sequence in O-linked neutral sugar chains of porcine zona pellucida glycoproteins (Hirano *et al.*, 1993). However, unlike the human zona pellucida, this sequence in pigs cannot be exposed by neuraminidase, and therefore does not react with PNA (Skutelsky *et al.*, 1994).

Owing to its carboxylic group, sialic acid constitutes a highly negatively charged moiety and, as such, largely contributes to the charge properties of cellular membrane and extracellular matrices. The possibility that the high content of sialylated oligosaccharides in the human zona pellucida might be expressed in a high density of anionic sites was studied using ruthenium red, a polycationic histochemical dye known to interact with carboxyl groups of sialyl residues (Luft, 1971; Skutelsky *et al.*, 1992). In agreement with a previous study (Familiari *et al.*, 1992) our results showed that this dye,

which positively stained some extracellular material on both sides of the zona pellucida, failed to reveal such sites in the matrix of the zona pellucida itself. Similar results were previously obtained in the zonae pellucidae of other mammals (Skutelsky *et al.*, 1992), indicating that, despite the high sialic acid content, the human zona pellucida is not negatively charged.

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