Cumulus Oophorus-associated Glycodelin-C Displaces Sperm-bound Glycodelin-A and -F and Stimulates Spermatozoa-Zona Pellucida Binding

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Philip C. N. Chiu1, Man-Kin Chung1, Riitta Koistinen4+, Hannu Koistinen1, Markku Seppala1, Pak-Chung Ho2†, Ernest H. Y. Ng1, Kai-Fai Lee1, and William S. B. Yeung1††

From the 1Department of Obstetrics and Gynaecology, University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong SAR, China and the Departments of 4Obstetrics and Gynaecology and 2Clinical Chemistry, University of Helsinki and Helsinki University, Central Hospital, 00029 HUS Helsinki, Finland

Spermatozoa have to swim through the oviduct and the cumulus oophorus before fertilization in vivo. In the oviduct, spermatozoa are exposed to glycodelin-A and -F that inhibit spermatozoa-zona pellucida binding. In this study, we determined whether these glycodelins would inhibit fertilization. The data showed that the spermatozoa without previous exposure to glycodelin-A and -F acquired glycodelin immunoreactivity during their passage through the cumulus oophorus. On the other hand, when glycodelin-A or -F-pre-treated spermatozoa were exposed to the cumulus oophorus, the zona pellucida binding inhibitory activity of glycodelin-A and -F was not removed, but the spermatozoa acquired enhanced zona pellucida binding ability. These actions of the cumulus oophorus were due to the presence of a cumulus isoform of glycodelin, designated as glycodelin-C. The cumulus cells could convert exogenous glycodelin-A and -F to glycodelin-C, which was then released into the surrounding medium. The protein core of glycodelin-C was identical to that in other glycodelin isoforms, as demonstrated by mass spectrum, peptide mapping, and affinity to anti-glycodelin antibody recognizing the protein core of glycodelin. In addition to having a smaller size and a higher isoelectric point, glycodelin-C also had lectin binding properties different from other isoforms. Glycodelin-C stimulated spermatozoa-zona pellucida binding in a dose-dependent manner, and it effectively displaced sperm-bound glycodelin-A and -F. In conclusion, the cumulus cells transform glycodelin-A and -F to glycodelin-C, which in turn removes the spermatozoa-zona binding inhibitory glycodelin isoforms and enhances the zona binding capacity of spermatozoa passing through the cumulus oophorus.

After ovulation, the oocyte-cumulus complex and follicular fluid are transported to the oviduct. Therefore, the spermatozoa have to traverse through the follicular fluid and the cumulus oophorus before they bind to the zona pellucida of the oocyte to initiate the fertilization process. Failure of cumulus oophorus formation results in problems in fertilization (1–4). Both the follicular fluid and the cumulus oophorus are known to modulate sperm function (5, 6). The cumulus oophorus may select spermatozoa with normal morphology (7, 8) and with intact acrosome (9) and create an optimal microenvironment to facilitate fertilization (7, 10).

Glycodelin is a lipocalin glycoprotein with three well known isoforms, namely glycodelin-A (amniotic fluid isoform), glycodelin-S (seminal plasma isoform), and glycodelin-F (follicular fluid isoform) (11, 12). These isoforms have molecular sizes of 28–30 kDa. They have the same protein core and only differ in their glycosylation (11, 13).

Glycodelin isoforms are present in the reproductive tract through which the spermatozoa must pass before fertilizing the oocyte. Different isoforms have diverse actions on sperm functions (13). Glycodelin-S maintains the spermatozoa in an uncapacitated state before their entry into the uterine lumen (14). Glycodelin-A and -F inhibit spermatozoa-zona pellucida binding (15–17). The latter, but not the former, inhibits progesterone-induced acrosome reaction, thus preventing premature acrosome reaction of spermatozoa (12, 18).

The fallopian tube produces glycodelin (19, 20) with increasing expression during the luteal phase (21). Our unpublished observation2 shows that human oviductal cells secrete both glycodelin-A and -F in vitro. Glycodelin-F is the main glycodelin isoform in the follicular fluid. Thus, spermatozoa are exposed to these glycodelins in the oviduct and follicular fluid before they fertilize an oocyte. It seems paradoxical that both glycodelin-A and -F inhibit the binding of spermatozoa to the zona pellucida. Whether these glycodelin isoforms would inhibit fertilization in vivo remains unclear.

Biological activities are often regulated by a fine balance between agonist and antagonist actions. For instance, progesterone induces acrosome reaction, but its activity is inhibited by human oviductal fluid (22) or glycodelin-F from human follic-
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Collection of Cumulus Oophorus and Cumulus Matrix—The Ethics Committee of the University of Hong Kong approved the protocol for collection and use of cumulus oophorus and cumulus matrix in this study. Cumulus-oocyte complexes were collected after oocyte retrieval from women who came for treatment with intracytoplasmic sperm injection because of male infertility. Human menopausal gonadotropin (Serono, Geneva, Switzerland) was used for ovarian stimulation after down-regulation with buserelin (27). For use in the capillary-cumulus model (6), intact cumulus oophorus was obtained by mechanical dissection from the cumulus-oocyte complex. The cumulus matrix was collected after dispersion of the cumulus oophorus in 1 ml of 0.1% hyaluronidase at 37 °C followed by centrifugation at 300 × g for 10 min to remove the cumulus cells.

Zona Binding Capacity of Spermatozoa Penetrating through the Cumulus Oophorus—A capillary-cumulus model was used (6). Briefly, EBSS/BSA and cumulus oophorus were successively aspirated into a sterile glass capillary (Microcaps; Drummond Broomall, PA) to form columns of 3 and 2 cm in length, respectively. The end of the capillary with the cumulus column was dipped into a 100-μl droplet of sperm suspension containing 10 × 10⁶ motile spermatozoa/ml overlaid with mineral oil. Another capillary containing only EBSS/BSA serves as the control. The setup was kept in a mobile in vitro fertilization chamber (Air-shields, Inc. Hatboro, PA) at 37 °C in an atmosphere of 5% CO₂ in air. After incubation for 1 h, the experimental capillary was cut at the interface between the cumulus column and the medium column. Spermatozoa that had passed through the cumulus oophorus (penetrated spermatozoa) were collected in the medium column. The control capillary was cut at a level that was similar to that of the experimental capillaries. Spermatozoa that had swum above the cutting level of the control capillary were collected as the control spermatozoa.

The hemizona binding assay described (28) was miniaturized to determine the zona binding capacity of the above spermatozoa. Unfertilized oocytes from assisted reproduction program were microbisected into two identical hemizonae. In this modified protocol, 6000 spermatozoa were incubated with each hemizona in 30 μl of EBSS/BSA under mineral oil for 3 h at 37 °C in an atmosphere of 5% CO₂ in air. The numbers of tightly bound spermatozoa on the outer surface of the hemizonae were counted. The hemizona binding index (HZI) was defined as the ratio of the number of bound spermatozoa in test droplet to that in the control droplet times 100. The experiment was repeated 10 times using different sperm samples and cumulus masses.

Glycodelin immunoreactivity on penetrated spermatozoa was determined by staining using monoclonal anti-glycodelin antibody (clone F43-7F9) recognizing the protein core of glycodelins (12). Staining without the primary antibody was used as control. The experiment was repeated five times with five semen samples. The presence of glycodelin immunoreactivity in these spermatozoa suggested the presence of glycodelin in the cumulus matrix (see “Results”). Western blot analysis of the cumulus matrix using the same antibody was used to confirm the observation.

Identification Glycodelin from Cumulus Matrix—Solubilized cumulus matrix was diluted 1:5 (v/v) with Tris-buffered saline.
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(TBS). The glycodelin in the matrix were isolated by the monoclonal anti-glycodelin antibody-Sepharose column as described above. The bound glycodelin was eluted and further fractionated by a Mono-Q column using a stepwise elution with 20 mM Tris-HCl, 0.5 mM NaCl (pH 7.0) at a flow rate of 100 μl/min. The eluted fractions were concentrated by Microcon-10 (Millipore). Their purities were determined by SDS-PAGE.

The bands in SDS-PAGE showing glycodelin immunoreactivity were excised for in situ digestion with trypsin (0.1 mg/ml in 25 mM NH₄HCO₃, pH 8.0). The peptides were recovered with Millipore C18 ZipTips and 60% acetonitrile with 0.1% trifluoroacetic acid containing α-cyano-4-hydroxycinnamic acid matrix. The peptide-matrix samples were analyzed with the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry to obtain the peptide mass spectra, which were compared with the protein spectra in the protein data bases at the Swiss-Prot (www.ebi.ac.uk/swissprot/) as described (29).

Biochemical Characterization of Cumulus Glycodelin—The protein core of cumulus glycodelin was also studied by peptide mapping (12). In this experiment, 600 pmol of deglycosylated glycodelin-A or -S or cumulus glycodelin was digested in 40 μl of 0.1 M NaHCO₃ with 2 units of agarose bound trypsin (Sigma) for 20 h at 37 °C. The resulting peptides in the supernatant were analyzed on a 15% SDS-PAGE.

The isoelectric point of the molecule was determined by analytical isoelectric focusing on 5% polyacrylamide gel (30) in a pH range of 3–10. The experiment was performed with 25 mM NaOH (cathode buffer) and 20 mM acetic acid (anode buffer) at 200 V for 1.5 h followed by 400 V for another 1.5 h. The protein bands were stained by Coomassie Blue, and their pI values were estimated using the IEF calibration kits (Amersham Biosciences).

Lectin binding assay (14) was used to study the glycosylation of cumulus glycodelin. Briefly, the wells in 96-well plate were coated with various lectins (10 μg/ml) overnight. Uncoated sites in the well were blocked by incubation with 100 μl of 10 mg/ml BSA in phosphate-buffered saline for 3 h with slow shaking. Glycodelins (7.5 pmol in 25 μl) were then added and incubated overnight at 4 °C. After washing the wells twice with 0.05% Tween 20-TBS, monoclonal anti-glycodelin antibody (clone F43-7F9, 2.5 μg in 200 μl) was added, and the mixture was incubated for 2 h. The wells were washed four times and 100 μl of horseradish peroxidase-conjugated anti-mouse IgG (Sigma) at a dilution of 1:300 was then added. The bound fluorescence was measured with a fluorometer (FL600; Bio-Tek).

Biological Characterization of Cumulus Glycodelin—The equilibrium binding of cumulus glycodelin to spermatozoa was determined as described (14). Four semen samples were used (n = 4). Spermatozoa (2 × 10⁵ motile spermatozoa/ml) in 100 μl of EBSS/BSA were incubated with different concentrations (0.162343 pmol/ml) of iodinated cumulus glycodelin at 37 °C for 1 h. The binding was terminated by the addition of 1.5 ml of ice-cold phosphate-buffered saline followed by centrifugation at 300 g for 3 min. After washing the spermatozoa with fresh EBSS/BSA, the sperm-bound radioactivity was counted with a gamma counter (model 5500B; Beckman, Fullerton, CA). The specific binding of cumulus glycodelin was determined by subtracting the sperm-bound radioactivity in the presence of a 50-fold higher concentration of unlabeled glycodelin from that in the absence of unlabeled protein. The determinations of total binding and nonspecific binding were done in duplicate.

Competition binding analysis was used to compare the affinity of cumulus glycodelin and other lipocalins to spermatozoa (14). The binding of 200 pmol/ml of 125I-cumulus glycodelin to 2 × 10⁵ motile spermatozoa (n = 3) in 100 μl of EBSS/BSA was determined in the presence of increasing concentrations (2, 20, 200, 1000, and 2000 pmol/ml) of unlabeled cumulus glycodelin, glycodelin-A, -F, and -S, deglycosylated glycodelin and bovine β-lactoglobulin A (Sigma), or buffer alone at 37 °C for 30 min. The cell-bound radioactivity was then determined. Each individual experiment was repeated twice.

The binding of purified cumulus glycodelin to spermatozoa was visualized by immunohistochemical staining after the incubation of capacitated spermatozoa (n = 5; 2 × 10⁵ spermatozoa/ml) with 200 pmol/ml cumulus glycodelin for 60 min at 37°C under 5% CO₂ in air. Binding of deglycosylated glycodelin was used as a control.

Hemizona binding assay was used to study the effect of cumulus glycodelin on spermatozoa–zona pellucida binding. Briefly, capacitated spermatozoa (2 × 10⁵ spermatozoa/ml; n = 5) were incubated with 0.3, 3, 30, 300, or 750 pmol/ml cumulus glycodelin or EBSS/BSA (control) at 37°C in an atmosphere of 5% CO₂ in air. After 1 h of incubation, the spermatozoa were washed with fresh EBSS/BSA. Standard protocol of hemizona binding assay (28) was performed on the 20,000 treated spermatozoa in 100 μl of EBSS/BSA. Glycodelin-A, -F, and -S and deglycosylated glycodelin were used as controls.

The effect of cumulus glycodelin on sperm motility, viability, and acrosomal status were also determined. Fluorescein isothiocyanate-labeled peanut (Pisum sativum) agglutinin (FITC-PSA; Sigma) and Hoechst staining techniques were used to evaluate the acrosomal status of spermatozoa (16). The fluorescence patterns of 300 spermatozoa in randomly selected fields were determined under a fluorescence microscope (Zeiss, Oberkochen, Germany) with 600× magnification. Spermatozoa without Hoechst and FITC-PSA staining or with FITC-PSA staining confined to the equatorial segment only were consid-
erated as acrosome reacted. Hobson Sperm Tracker System (Hobson Tracking Systems Ltd., Sheffield, UK) was used to study sperm motility. The set-up parameters of the system and the procedures are described elsewhere (12).

**Effects of Cumulus Penetration on the Zona Binding Capacity of Glycodelin-A- and -F-treated Spermatozoa**—Capacitated spermatozoa (2 × 10⁶ spermatozoa/ml) were incubated with 300 pmol/ml of glycodelin-F or -A, deglycosylated glycodelin or EBSS/BSA (control) at 37 °C in an atmosphere of 5% CO₂ in air for 2 h. They were then washed and allowed to penetrate the cumulus oophorus column in the capillary as described above. The penetrated spermatozoa were washed with fresh EBSS/BSA, and their zona binding capacities were determined by hemizona binding assay. The experiment was repeated 10 times with different sperm samples and cumulus masses.

The fate of sperm-bound glycodelin-F and -A after cumulus oophorus penetration was investigated by repeating the above experiment using iodinated or Alexa 488 (Alexa Fluor® 488 protein labeling kit; Molecular Probes, Carlsbad, CA)-conjugated glycodelin-F and -A. The penetrated spermatozoa were collected and either smeared on slides to observe under a fluorescence microscope with an excitation filter BP 450–490, chromatic beam splitter FT510, and barrier filter LP520 or counted for their associated radioactivity as above.

**Effects of Cumulus Glycodelin on Sperm-bound Glycodelin-A and -F**—Iodinated or Alexa 488-conjugated glycodelin-A or -F (300 pmol/ml) were incubated with 2 × 10⁵ motile spermatozoa (n = 3) in 100 µl of EBSS with 0.3% BSA at 37 °C for 120 min. Unlabeled glycodelin-A or -F, cumulus glycodelin, or deglycosylated glycodelin (200 pmol/ml) was then added. After a further incubation for 0, 2, 5, 10, 15, 20, 30, 60, or 90 min, ice-cold buffer was added to stop the dissociation. The spermatozoa were then washed, and their associated radioactivity or fluorescence was determined as described above.

**Processing of Glycodelin by Cumulus Cells**—The dispersed cumulus cells were washed twice in TC-199 medium (Sigma) and resuspended to a final concentration of 4 × 10⁶ viable cells/ml. Trypan blue exclusion test was employed to determine the viability of the cells. They were then cultured in 2 ml of TC-199 medium supplemented with 20% fetal bovine serum (Sigma) as described (31). After 24 h, 300 pmol/ml glycodelin-A or -F was added to the culture medium. The spent media were collected after 10 h and diluted 1:5 (v/v) with TBS. 94% of the cells remained viable at this time. The cell debris in the conditioned media was removed by centrifugation at 500 × g for 5 min. The glycodelin in the media were immunoaffinity-purified using monoclonal anti-glycodelin antibody-Sepharose column. The eluates from 10 runs were combined and analyzed by 15% SDS-PAGE.

**Data Analysis**—All of the data were expressed as the means ± S.E. The data were analyzed by statistical softwares (SigmaPlot 8.02; Ligand Binding Analysis Module & SigmaStat 2.03, Jandel Scientific, San Rafael, CA). For all experiments, the nonparametric analysis of variance on Rank test for multiple comparisons followed by Mann Whitney U test were used. A probability value <0.05 was considered to be statistically significant.

**RESULTS**

**Cumulus Oophorus-penetrated Spermatozoa Had Increased Zona Binding Capacity and Glycodelin Immunoreactivity**—Eight semen samples were recruited. By comparing with spermatozoa in the control capillary, the cumulus oophorus penetrated spermatozoa had a higher zona binding capacity with the number of tightly bound spermatozoa on the hemizona (72.6 ± 5.6), significantly higher than that of the control spermatozoa (58.3 ± 6.2), and the HZI was increased from 112.4 ± 3.8 to 136.4 ± 6.2. Indirect immunofluorescence staining revealed glycodelin immunoreactivity on the whole head of cumulus penetrated spermatozoa, the signal being stronger on the equatorial region (Fig. 1A). This staining pattern was different from that of the glycodelin-A or -F-treated spermatozoa (12). The percentages of spermatozoa with positive immunoreactivity were ~82–94%. In contrast, no immunoreactivity was observed on spermatozoa in the control capillary or with the omission of the primary antibody (data not shown).

**Cumulus Matrix Contains a Glycodelin Isoform**—The above experiment suggested the presence of glycodelin in the cumulus matrix. This was confirmed by Western blot analysis of the extracellular matrix of cumulus oophorus using anti-glycodelin antibody. The data revealed two major bands of sizes ~30 and ~25 kDa. The molecular size of the larger band was similar to that of glycodelin-A (Fig. 1B) and -F (12).

Liquid chromatography was used to isolate the glycodelins in the cumulus matrix. A total of 250 cumulus samples were used for the purification. A typical ion exchange chromatogram of the eluate of cumulus matrix components bound to anti-glycodelin-Sepharose column is shown in Fig. 2A. There were three major peaks, named fractions F1, F2, and F3 according to their ionic strength. SDS-PAGE showed that all fractions contained a major protein band of sizes larger than that of the deglycosylated glycodelin-A or -F (Fig. 2B, lanes 4 and 5). The molecular masses of F1 (Fig. 2B, lane 1) and F2 (Fig. 2B, lane 2) were similar to that glycodelin-A and -F (~30 kDa). Based on their mass spectrometry pattern, ionic strength, molecular weights, lectin binding properties, and immunoreactivity (data not shown), F1 and F2 were glycodelin-A and -F, respectively. F3 contained the cumulus glycodelin with size ~25 kDa that...
was intermediate between glycodelin-A and -F and their deglycosylated form (Fig. 2B, lane 3). After glycosidase digestion (Fig. 2C), the molecular size of F3 decreased to that of deglycosylated glycodelin-A and -F (Fig. 2C, lanes 2, 4, and 7).

**Biochemical Characteristics of Cumulus Glycodelin—**Mass spectrometry analysis of the tryptic digests and data base search identified the ~25-kDa anti-glycodelin-bound protein as glycodelin. The eight-peptide sequences showed >93% match to the mass spectrometry spectra and confirmed the protein identity at a high confidence level (data not shown). After proteolytic digestion with trypsin, deglycosylated glycodelin-A and -F and cumulus glycodelin gave identical banding patterns (data not shown).

The yield of cumulus glycodelin obtained was 126 ± 28 pmol/patient. After accounting for loss during purification, each cumulus sample was estimated to contain 161 ± 36 pmol of cumulus glycodelin. The purity of isolated cumulus glycodelin is greater than 95% as determined by SDS-PAGE.

The dilution curves of the immobilized glycodelin-A and -F and F3 with limiting concentration of the anti-glycodelin antibody were similar (r² = 0.95; Fig. 3). This indicated that the cumulus glycodelin had similar immunoreactivity with the other glycodelin isoforms.

The net charges in cumulus glycodelin were different from those of glycodelin-A and -S. After isoelectric focusing, cumulus glycodelin was separated into several bands with pls of 5.2–6.0, whereas glycodelin-S and -A yielded bands with pls between 4.7–5.5 and 4.4–5.1, respectively (data not shown). The latter results were similar to that of a previous report (32).

The lectin binding properties of the cumulus glycodelin were different from those of glycodelin-F, -A, and -S (Table 1). Cumulus glycodelin reacted strongly with concanavalin A, *Wisteria floribunda* agglutinin, *Ricinus communis* agglutinin, *Ulex europaeus* agglutinin, and *Dolichos biflorus* agglutinin but not with the other lectins tested.

**Binding of Cumulus Glycodelin to Spermatozoa—**The specific binding of 125I-cumulus glycodelin to spermatozoa increased with the concentration of cumulus glycodelin up to 200 pmol/ml, after which no further increase was observed (Fig. 4). This result indicated that the binding was saturable. The Scatchard plot for the binding had a convex shape (R² > 0.92), suggesting a positive cooperative binding mechanism in which the binding of a ligand to one site increases the binding affinity for a second site (Fig. 4, inset A). The Hill coefficient from the saturation binding data were greater than 1 (1.5 ± 0.14), strengthening the concept of positive cooperativity (Fig. 4, inset B).

The purified cumulus glycodelin bound to both the acrosomal and the equatorial regions of the sperm head (Fig. 5). Using the same antibody, the pattern of immunoreactivity was different from that in the spermatozoa recovered after cumulus
TABLE 1

Binding of lectins with cumulus glycodelin and glycodelin-F, -A, and –S

The data are presented as the means ± S.E. (n = 5). The control coated with 10,000 ng/ml BSA instead of lectins had A values between 0.07 and 0.13.

<table>
<thead>
<tr>
<th>Lectin (specificity)</th>
<th>Lectin immunoassay (A405 ± S.E.)</th>
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<tr>
<td></td>
<td>F3</td>
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<tr>
<td>Peanut agglutinin (β-gal[1–3]GalNAc)</td>
<td>0.68 ± 0.01*a</td>
</tr>
<tr>
<td>Succinylated wheat germ agglutinin ([GlcNAc or its oligomer)</td>
<td>0.41 ± 0.08*a</td>
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<tr>
<td>Concanavalin A (α-Man, α-glc)</td>
<td>1.26 ± 0.18</td>
</tr>
<tr>
<td>W. floribunda agglutinin (GalNAc)</td>
<td>1.54 ± 0.10*a</td>
</tr>
<tr>
<td>R. communis agglutinin (β-gal)</td>
<td>1.47 ± 0.02*a</td>
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<tr>
<td>Sambucus nigra bark agglutinin (α-NeuNAc[2–6]gal/GalNAc)</td>
<td>0.67 ± 0.07*a</td>
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<tr>
<td>Wheat germ agglutinin ([GlcNAc]₆, NeuNAc)</td>
<td>0.36 ± 0.17*a</td>
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<tr>
<td>Limulus polyphemus agglutinin (NeuNAc[GalNAc, GlcNAc])</td>
<td>0.24 ± 0.09*a</td>
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<tr>
<td>U. europaeus agglutinin (α-1-fuc)</td>
<td>1.55 ± 0.06*a</td>
</tr>
<tr>
<td>D. biflorus agglutinin (GalNAc)</td>
<td>1.03 ± 0.29*a</td>
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*a** p < 0.05 within the same column (analysis of variance on rank).

FIGURE 4. Saturation binding of ¹²⁵I-cumulus glycodelin to human spermatozoa. Each point represents the mean ± S.E. of four experiments performed in duplicate. A, Scatchard plot; B, Hill plot of cumulus glycodelin from the saturation curve.

Specific Binding of Cumulus Glycodelin to Human Spermatozoa—The results of competitive binding to human spermatozoa between ¹²⁵I-cumulus glycodelin and β-lactoglobulin, another lipocalin protein, are shown in Fig. 6. Unlabeled cumulus glycodelin inhibited the binding of ¹²⁵I-cumulus glycodelin in a dose-dependent manner with a half-maximal inhibition (IC₅₀) of 40.79 ± 1.39 pmol/ml. However, the inhibition was only ~80% even when the concentration of unlabeled cumulus glycodelin was 10-fold greater than that of ¹²⁵I-cumulus glycodelin. Glycodelin-A and -F inhibited the binding only at high concentrations (IC₅₀ > 2000 pmol/ml), whereas the other lipocalin proteins tested did not affect the binding of ¹²⁵I-cumulus glycodelin to spermatozoa at all (IC₅₀ > 20000 pmol/ml).

Cumulus Glycodelin Dose-dependently Stimulated Spermatozoa-Zona Pellucida Binding—Cumulus glycodelin at concentrations >30 pmol/ml significantly increased the number of spermatozoa bound to the hemizona (p < 0.05) as compared with the control (Fig. 7). The HZI increased in a dose-dependent manner, and the corresponding stimulation at 750 pmol/ml was 174.8 ± 5.5%. In contrast, glycodelin-A and -F reduced the number of spermatozoa bound to hemizonae at concentration 0.3 pmol/ml or above. No effects were observed with glycodelin-S and deglycosylated glycodelin. At the concentrations employed, none of glycodelin isoforms affected sperm viability, acrosomal status, and motility (data not shown).

Cumulus Cell Passage Reduced Sperm-bound Glycodelin-A and -F—Spermatozoa were treated with glycodelin-A and -F and deglycosylated glycodelin and were subsequently allowed to swim through the capillary containing cumulus oophorus. In the control (capillary without cumulus oophor)us), glycodelin-A and -F, but not deglycosylated glycodelin, significantly decreased the number of spermatozoa bound to the hemizona as compared with the control without glycodelin. The HZIs were reduced to 61.9 ± 4.6, 49.0 ± 5.0 and 44.4 ± 4.0% for glycodelin-A and -F, respectively (Fig. 8), consistent with previous observations (12). After cumulus penetration, the HZIs of glycodelin-A and -F-treated groups were 131.6 ± 5.5 and 123.6 ± 4.6, respectively. This indicated that cumulus penetration not only removed the zona binding inhibitory activity of glycodelin-A and -F, but it also stimulated the zona binding capacity of the penetrated spermatozoa. The HZI of penetrated spermatozoa preincubated with deglycosylated glycodelins was 125.5 ± 5.6.
After the cumulus penetration, sperm-bound $^{125}$I-glycodelin-A and -F were significantly reduced ($p < 0.05$) as compared with the control spermatozoa in the capillary without cumulus oophorus (Fig. 9A); the radioactivity of glycodelin-A and -F-pretreated spermatozoa after cumulus penetration was only 35.4 ± 7.0 and 51.8 ± 6.4% of the control, respectively. This decrease in sperm-bound glycodelin was also visualized using fluorescence-labeled glycodelin isoforms (Fig. 9B). No binding was observed when iodinated or Alexa 488-conjugated deglycosylated glycodelin was used.

The displacement of sperm-bound $^{125}$I-glycodelin-A and -F by cumulus glycodelin is shown in Fig. 10A. The addition of 200 pmol/ml cumulus glycodelin rapidly decreased the specific binding of $^{125}$I-glycodelin-A and -F during the first 30 min of coincubation at 37 °C. The bound radioactivity was 25.7 ± 3.5 and 35.7 ± 3.2% of the maximum binding, respectively, after 90 min. The efficiency of displacement mediated by cumulus glycodelin was much higher than that by glycodelin-A or -F. Deglycosylated glycodelin was not effective in displacing sperm-bound glycodelin-A or -F. The reduction in sperm-bound glycodelin isoforms was also visualized when fluorescence-labeled molecules were used (Fig. 10B).

**Cumulus Cells Produced Cumulus Glycodelin**—The cumulus cells were incubated with glycodelin-A or -F at 37 °C for 10 h. Components in the spent medium with glycodelin immunoreactivity were isolated by anti-glycodelin affinity chromatography and analyzed on SDS-PAGE (Fig. 11). Apart from the glycodelin-A or F band, there is one additional band in the conditioned medium from the cumulus cells (Fig. 11, lanes 1 and 2). This band was absent in the medium derived from a fibroblast culture (Fig. 11, lanes 3 and 4) and from cumulus cells/fibroblasts without glycodelin treatment (Fig. 11, lanes 7 and 8). The additional band had a molecular size similar to that
discussed elsewhere (23). This study also demonstrates that the presence of glycodelin in the follicular fluid is more complex than previously thought.

**DISCUSSION**

Human follicular fluid reduced the zona binding capacity of human spermatozoa in vitro (16, 17, 33). Glycodelin-F is the main factor contributing to the inhibitory activity of human follicular fluid (12), although glycodelin-A is also present in the follicular fluid in a much lesser amount. These observations are controversial to the clinical observations that follicular fluid improves the results of in vitro fertilization (34), gamete intrafallopian transfer (35), intrauterine insemination (36), and subzonal sperm injection (37).

The present study provides an explanation for these contradictory observations, i.e. the cumulus oophorus not only removes the inhibitory activity of glycodelin-A and -F, but it modifies these isoforms to produce a cumulus glycodelin that stimulates spermatozoa-zona pellucida binding.

Spermatozoa have to traverse the cumulus oophorus to reach the oocyte. Therefore, the cumulus oophorus is at an ideal position to modulate the fertilizing ability of the spermatozoa passing through it. Indeed, the cumulus oophorus is known to affect human sperm function (7, 38–42) and select morphologically normal spermatozoa for fertilization (34). Cumulus cells also reduce the spermatozoa-zona binding inhibitory activity of human follicular fluid (23). Removal of the cumulus cells reduces the fertilization rate of oocyte (44–48) and the cleavage rate of the resulting embryo (49).

The action of the cumulus oophorus on sperm function may be mediated via secretory products of the cells or proteins from oviductal and follicular fluid that are trapped within the matrix (50–52). Conditioned medium from cumulus cell culture induces acrosome reaction (40) and stimulates sperm motility (41). In this study, the acquisition of glycodelin immunoreactivity after penetration of the cumulus oophorus and Western blot analysis of the cumulus matrix demonstrate the presence of a small glycodelin-like molecule that interacts with the spermatozoa. In addition to being smaller in size, this molecule also differs from the other glycodelin isoforms with respect to its carbohydrate moieties, surface charge, pI values, and binding pattern on human spermatozoa. On the other hand, the protein core of the molecule...
The surface of cumulus has not been explored. These possibilities include membrane of many cell types (56–59), although their presence on these observations, it is reasonable to name this molecule as glycodelin-C (cumulus matrix isoform of glycodelin) to be in agreement with the existing nomenclature of glycodelin. The luteinized granulosa cells but not the cumulus cells possess glycodelin mRNA, suggesting that the former is the site of biosynthesis of glycodelin (24). However, the cumulus cells can take up specifically exogenous glycodelin but not the other lipocalin family members, thereby modifying the uptake glycodelin to glycodelin-C (24). The present study confirms these previous observations and further demonstrates that the glycodelin-C herein produced is released back into the culture medium. Cumulus cells contain cytoplasmic glycodelin immunoreactivity but not glycodelin mRNA (24). Therefore, it is likely that exogenous glycodelin is internalized in cumulus cells probably by cell surface receptor-mediated endocytosis similar to that found in other lipocalin family members (53, 54). Cumulus cells have well developed machinery for glycan manipulation and produce plenty of carbohydrate-containing extracellular matrix molecules, such as proteoglycan (55), at the periovulatory period. Thus, the conversion of exogenous glycodelin to glycodelin-C is likely to occur intracellularly by a mechanism yet to be defined. However, these observations do not exclude the possibility that part of the conversion could be done on the plasma membrane of the cumulus cells. Glycosydrolase activities have been demonstrated on the plasma membrane of many cell types (56–59), although their presence on the surface of cumulus has not been explored. These possibilities are under investigation in our laboratory.

This is the first report on characterization of the binding of glycodelin-C to human spermatozoa. The binding is specific, and it is time- and concentration-dependent. Three observations indicate that the binding sites for glycodelin-C are different from those of the other glycodelin isoforms (14, 18, 60). First, the equilibrium binding of glycodelin-C to spermatozoa suggests a positive cooperative binding mechanism that does not occur in the binding glycodelin-A, -F, and -S to spermatozoa. Second, the binding sites of glycodelin-A and -F are found evenly on the acrosomal region (12), whereas those of glycodelin-C have differential distribution with the highest density and affinity at the equatorial region. The binding sites of glycodelin-S spread evenly over the whole head and had a low affinity for glycodelin-S (14). Third, the other glycodelin isoforms cannot significantly compete with glycodelin-C for its binding sites in the competition binding assay. Likewise, another lipocalin family member, β-lactoglobulin, is ineffective in competing with glycodelin-C.

In this study, the binding pattern of purified glycodelin-C on spermatozoa is different from that of glycodelin immunoreactivity on spermatozoa that had penetrated the cumulus oophorus; the former is confined to the acrosome and equatorial region, whereas the latter also includes the post-acrosomal region. The question of how the cumulus oophorus modifies the binding of glycodelin to spermatozoa is so far unknown. The hyaluronan, proteoglycans, and proteins in the cumulus extracellular matrix (55) modulate protein-receptor binding (61, 62). Therefore, it is possible that the cumulus oophorus induces the exposure and/or functioning of glycodelin receptors on the post-acrosomal region of spermatozoa. This hypothesis is under investigation in our laboratory.

Interestingly, treatment of spermatozoa with either glycodelin-A or -F prior to cumulus penetration did not affect the zona pellucida binding capacity of the penetrated spermatozoa. In fact, cumulus penetration has two effects on the penetrated spermatozoa, namely removal of the inhibitory effect of glycodelin-A and -F and stimulation of the zona binding capacity of the penetrated spermatozoa. The former effect is consistent with significant reduction of sperm-bound glycodelin-A and -F. Glycodelin-C is likely to be at least partly responsible for reducing the bound glycodelin isoforms because it could effectively displace sperm-bound glycodelin-A and -F to ~30 and 40% of the maximum binding, respectively, after 60 min. Indeed, its displacement activity is more effective than that of glycodelin-A or -F. An efficient mechanism for removal of bound glycodelin-C and -F is likely to be important for fertilization, because human spermatozoa can penetrate the cumulus oophorus within 1 h in vitro (6, 63, 64).

Glycodelin-C is also likely to be responsible for the second effect of cumulus penetration on the penetrated spermatozoa because it stimulates spermatozoa zona-pellucida binding in a dose-dependent manner. No other glycodelin isoform has a similar stimulatory activity. The stimulatory activity of glycodelin-C is not due to the effect on acrosome reaction and sperm motility, and it is no exception to the general observation that the activity of glycodelin isoforms on spermatozoa-zona pellucida interaction is glycosylation-dependent (12, 32, 65). Glyco-

**FIGURE 11. Detection of cumulus glycodelin in condition medium of cumulus cells by anti-glycodelin affinity chromatography and 15% SDS-PAGE.** Lane 1, conditioned medium of human cumulus cells after glycodelin-A treatment; lane 2, conditioned medium of human cumulus cells after glycodelin-F treatment; lane 3, conditioned medium of human fibroblast after glycodelin-A treatment. lane 4, conditioned medium of human fibroblast after glycodelin-F treatment; lane 5, purified cumulus glycodelin; lane 6, purified glycodelin-A; lane 7, conditioned medium of human cumulus cells without glycodelin treatment; lane 8, conditioned medium of human fibroblast without glycodelin treatment.
Glycodelin and Spermatozoa

Carbohydrate recognition is important in spermatozoa-zona pellucida binding, although the details of the molecular interactions between sperm membrane and zona pellucida proteins remain elusive (66–68). The zona binding stimulatory activity of glycodelin-C suggests that changes in glycosylation of glycodelin can have significant effects on spermatozoa-zona pellucida binding. Comparison of the glycosylation of different glycodelin isomers is ongoing to identify the role of different glycans in the regulation of spermatozoa-zona pellucida binding. Modification of biological activity resulting from the changes in glycosylation has been demonstrated in different glycohormones and glycoproteins (69).

How glycodelin-C stimulates spermatozoa-zona binding is largely unknown. It may act as a "bridge" between spermatozoa and zona pellucida, using its unique carbohydrate moieties to promote binding between the sperm membrane receptor(s) and the zona pellucida proteins. A recent report demonstrated that when glycodelin binds to its receptor on T cells, CD45, it can act as a calcium-dependent lectin to bind other T cell surface glycoproteins to mediate its immunoregulatory activities (70). Gonadotropin-releasing hormone increases spermatozoa-zona binding via an elevation of intracellular calcium through T-type voltage-operated calcium channels (71). It is also possible that glycodelin-C induces signal transduction leading to the formation of more functional zona pellucida receptors. In mouse, tyrosine phosphorylation-associated change of endoplasmin and heat shock protein 60 in spermatozoa-zona binding receptors. In mouse, tyrosine phosphorylation-associated change of endoplasmin and heat shock protein 60 in spermatozoa facilitates the formation of functional zona pellucida receptors, resulting in increased zona pellucida binding ability (72). These possibilities are being investigated in our laboratory.

Based on the results of this report and previous results, we propose that the following sequence of events take place at the fertilization site: Glycodelin is synthesized in the granulosa cells of the late secondary follicle. During the periovulatory period, the cumulus cells use glycodelin-A and -F in the follicular fluid as substrate for the production of the glycodelin-C, which then is released into the extracellular matrix during cumulus expansion or shortly after ovulation. Glycodelin-F and probably oviductal-derived glycodelin (19) binds to spermatozoa and protects them from premature acrosome reaction. During cumulus penetration, glycodelin-C in the matrix displaces sperm-bound glycodelin isoforms and promotes the zona binding capacity of the penetrated spermatozoa. Besides, the removal of glycodelin-F may allow a more efficient zona-induced acrosome reaction to occur on the surface of the zona pellucida.

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Cumulus Oophorus-associated Glycodelin-C Displaces Sperm-bound Glycodelin-A and -F and Stimulates Spermatozoa-Zona Pellucida Binding

Philip C. N. Chiu, Man-Kin Chung, Riitta Koistinen, Hannu Koistinen, Markku Seppala, Pak-Chung Ho, Ernest H. Y. Ng, Kai-Fai Lee and William S. B. Yeung

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