Expression of a P-selectin Ligand in Zona Pellucida of Porcine Oocytes and P-selectin on Acrosomal Membrane of Porcine Sperm Cells. Potential Implications for Their Involvement in Sperm–Egg Interactions

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Abstract. The selectin family of cell adhesion molecules mediates initial leukocyte adhesion to vascular endothelial cells at sites of inflammation. O-glycan structural similarities between oligosaccharides from human leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) and from zona pellucida glycoproteins of porcine oocytes indicate the possible existence of a P-selectin ligand in the zona pellucida. Here, using biochemical as well as morphological approaches, we demonstrate that a P-selectin ligand is expressed in the porcine zona pellucida. In addition, a search for a specific receptor for this ligand leads to the identification of P-selectin on the acrosomal membrane of porcine sperm cells. In vitro binding of porcine acrosome-reacted sperm cells to oocytes was found to be Ca²⁺ dependent and inhibitable with either P-selectin, P-selectin receptor–globulin, or leukocyte adhesion blocking antibodies against P-selectin and PSGL-1. Moreover, porcine sperm cells were found to be capable of binding to human promyeloid cell line HL-60. Taken together, our findings implicate a potential role for the oocyte P-selectin ligand and the sperm P-selectin in porcine sperm–egg interactions.

Cell–cell and cell–matrix interactions play central roles in biological development, such as during fertilization, implantation, placenta formation, embryogenesis, cell differentiation, migration, and organ formation. These types of interactions are also essential for a variety of physiological and pathological processes, such as lymphocyte trafficking, immune defense, hemostasis, wound healing, cancer cell invasion, and metastasis. Cell–cell and cell–matrix interactions are precisely controlled and regulated by cell and matrix adhesion molecules with specificities appropriate for their particular functions (Gumbiner, 1992; Hynes and Lander, 1992; Cross et al., 1994; Wassarman, 1995; Snell and White, 1996).

Recruitment of leukocytes, from the flowing blood stream across the endothelial cells of postcapillary venules into the tissue at sites of inflammation or injury, is a multistep paradigm requiring at least three sequential steps. Three major families of cell adhesion molecules are involved in this process: selectins, integrins, and adhesion molecules of the immunoglobulin superfamily (Butcher, 1991; Lasky, 1992; Springer, 1994). The selectins comprise a subfamily of Ca²⁺-dependent (C-type) animal lectins (Drickamer, 1988, 1993) that are mainly responsible for the initial leukocyte tethering to, and rolling on, the activated endothelial cells. Three members of this family have been described to date (Butcher, 1991; Lasky, 1992; Springer, 1994). L-selectin (CD62L) is a constitutively expressed homing receptor on a majority of leukocytes for lymphatic and vascular endothelial cells. E-selectin (CD62E) is a cytokine-inducible cell surface receptor on vascular endothelial cells, and P-selectin (CD62P) is a rapidly inducible receptor on vascular endothelial cells and platelets. E- and P-selectins function as cell surface receptors for neutrophils, monocytes, T lymphocyte subsets, eosinophils, and basophils.

All three selectins recognize a sialoglycoprotein ligand, P-selectin glycoprotein ligand-1 (PSGL-1) (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995; Spertini et al., 1996; Tu et al., 1996). PSGL-1 is a disulfide-linked dimeric sialomucin expressed on the microvilli of human leukocytes. The recognition of PSGL-1 by the three selectins is Ca²⁺ dependent and sialidase sensitive, characteristics of selectin-mediated leuko-

1. Abbreviations used in this paper: BCECF-AM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; PGSL-1, P-selectin glycoprotein ligand-1; PUVEC, porcine umbilical vein endothelial cell; Rg, receptor-globulin; TNF-α, tissue necrosis factor-α.
cyte adhesion (Rosen et al., 1985; Yednock and Rosen, 1989; Bevilacqua et al., 1987, 1989; Larsen et al., 1989; Geng et al., 1990).

All three selectins have been reported to bind to the tetrasaccharide structure NeuNAcc2-3Galβ1-4(Fucα1-3) GlcNAc (called sialyl Lewis x or SLeα) and its isomer, NeuNAcc2-3Galβ1-3(Fucα1-4)GlcNAc (called sialyl Lewis a or SLeα); these structures may constitute the minimal recognition motif for the three selectins (Brandley et al., 1990; Varki, 1994). In a previous investigation, we isolated PSGL-1 from [3H]glucosamine-labeled HL-60 cells, a human promyeloid cell line, by P- and E-selectin affinity chromatography. The desialylated O-linked oligosaccharides released from this molecule were separated into five well-defined peaks having elution volumes corresponding to glucose oligomers composed of 2.5, 3.5, 6.3, 9.8, and 12.8 glucose units, respectively (Asa et al., 1995). Sequencing of the carbohydrate structures in these peaks resulted in the identification of a set of oligosaccharides (Aeed, P., manuscript submitted for publication), which had considerable similarities to structures previously identified on glycoproteins isolated from the porcine zona pellucida (Hirano et al., 1993).

The involvement of oligosaccharide structures in mammalian gamete interactions is well documented (Florman et al., 1993). In a previous investigation, we isolated PSGL-1 from HL-60 cells, a human promyeloid cell line, by P- and E-selectin affinity chromatography. The desialylated O-linked oligosaccharides released from this molecule were separated into five well-defined peaks having elution volumes corresponding to glucose oligomers composed of 2.5, 3.5, 6.3, 9.8, and 12.8 glucose units, respectively (Asa et al., 1995). Sequencing of the carbohydrate structures in these peaks resulted in the identification of a set of oligosaccharides (Aeed, P., manuscript submitted for publication), which had considerable similarities to structures previously identified on glycoproteins isolated from the porcine zona pellucida (Hirano et al., 1993).

Preparation of Porcine Oocytes and Zona Pellucida

Porcine oocytes and zona pellucida were prepared from frozen or fresh ovaries essentially as described (Dunbar et al., 1989). No obvious contamination by leukocytes could be observed by microscopy. The concentration of total zona pellucida proteins was determined by amino acid compositional analysis.

Isolation of Porcine Sperm Cells

Fresh porcine sperm were collected at a local farm and kept at 37°C until use. No obvious contamination of blood cells could be found in the white milky suspension. The sperm was kept still for at least 15 min at 37°C in the presence of 5% CO₂, and “swim-up” sperm cells were carefully collected on the top layer of the sperm suspension. This was done to avoid the possible contaminating blood cells and to eliminate less viable sperm cells. No obvious contamination by leukocytes could be observed by microscopy.

Preparation of Porcine Umbilical Vein Endothelial Cells

Porcine umbilical vein endothelial cells (PVVEC) were prepared from the freshly collected porcine umbilical cords exactly as previously described for human umbilical vein endothelial cells (Geng et al., 1990; Ma et al., 1994). For induction of E-selectin expression, confluent monolayers of PVVEC (third passage) were treated with 300 U/ml of TNF-α at 37°C for 4 h. After washing once with PBS, the cells were harvested by mechanical detachment with a cell scraper (Nunc, Naperville, IL) in the presence of Versene™ (GIBCO BRL, Gaithersburg, MD).

CHO Cell Line Expressing E-Selectin

A stable CHO-K1 cell line expressing human full-length E-selectin was established by cotransfection of E-selectin cDNA in CDMS vector (10 μg/ml) with pCDNA1/Neo (1 μg/ml) using a Lipofectin™ (GIBCO BRL) method according to manufacturer’s protocol. 3 d later, CHO cells were selected and maintained in DME (high glucose) in the presence of 10% FCS (vol/vol) and 0.4 μg/ml of active Gentamicin™ (wt/vol; GIBCO BRL).
SDS-PAGE and Silver Staining

Aliquots of total zona pellucida proteins (~38 μg per lane) and sperm cells (5 × 10⁶ cells per lane; washed three times with ice-cold PBS) were mixed with SDS sample buffer in the presence or absence of 5% β-mercaptoethanol (vol/vol). After boiling for 5 min, samples were subjected to 7% SDS-PAGE. After electrophoresis, proteins were silver stained (Bio Rad Laboratories, Hercules, CA).

Ligand Blotting

Aliquots of total zona pellucida proteins (~115 μg per lane) and porcine leukocyte membrane extracts (~500 μg per lane) were mixed with SDS sample buffer in the presence or absence of 5% β-mercaptoethanol (vol/vol). Samples were boiled for 5 min and subjected to 7% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). The membranes were probed with P-selectin followed by biotinylated P23 mAb (1 μg/ml). The membranes were subsequently incubated with a streptavidin–peroxidase complex (Vectorstain ABC kit; Vector Laboratories, Burlingame, CA). A chemiluminescent detection system (Amersham Corp., Arlington Heights, IL) was used for detection (Ma et al., 1994; Asa et al., 1995). All incubation and washing buffers contained either 1 mM CaCl₂ or 2 mM EDTA as indicated.

Immunoblotting

Aliquots of total zona pellucida proteins (~115 μg per lane), sperm cells (1 × 10⁷ cells per lane), and TNF-α-treated PUV Ecs (confluent monolayer of cells from a 35-mm dish per lane) were subjected to 7% SDS-PAGE. The separated proteins were transferred to the blotting membranes as described above. For the zona pellucida proteins, the blotting membranes were probed with preimmune IgG or PSGL-1 peptide antibody (both at 1 μg/ml). They were then incubated with biotinylated goat anti-rabbit IgG antibodies against rabbit IgG (5 μg/ml). For sperm and PUV Ecs, the blotting membranes were probed separately with either biotinylated rabbit P- or E-selectin antibody or biotinylated P7 mAb (all at 1 μg/ml). The membranes were subsequently incubated with a streptavidin–peroxidase complex followed by a chemiluminescent detection system as described above.

Flow Cytometric Analysis

Porcine sperm cells were used either unwashed or washed three times with HBSS/FCS (1% heat-inactivated FCS in HBSS; vol/vol; 3,000 rpm for 10 min). More than 90% of the washed sperm cells were mechanically capacitated, as determined using Coomassie brilliant blue (Aarons et al., 1991). The sperm cells were resuspended in HBSS/FCS (2 × 10⁶ cells per ml) and incubated separately with either an FITC-conjugated rabbit preimmune IgG, an FITC-conjugated rabbit polyclonal antibody against P- or E-selectin, an FITC-conjugated mouse preimmune IgG, or an FITC-conjugated P7 mAb (all at 10 μg/ml) at 22°C for 1 h. For staining of PUV Ecs, mechanically detached cells (confluent monolayer of cells from a 35-mm dish per aliquot) were resuspended in HBSS/FCS and incubated with an FITC-conjugated rabbit preimmune IgG or an FITC-conjugated rabbit P-selectin antibody, as outlined above. After incubation, the cells were sedimented by centrifugation at 1,500 rpm for 5 min and washed twice with HBSS/FCS. The cells were then resuspended in HBSS/FCS for flow cytometric analysis (FACScan®, Becton Dickinson & Co., Mountain View, CA).

Immunoelectron Microscopy

Porcine oocytes were washed with HBSS/FCS and incubated with purified platelet P-selectin (10 μg/ml) at 22°C for 1 h. As controls, P-selectin was either omitted or the oocytes were incubated with P-selectin in calcium and magnesium-free HBSS/FCS containing 2 mM EDTA. After washing, the oocytes were incubated with rabbit P-selectin antibody (25 μg/ml) for 30 min. For antibody staining, porcine oocytes were first incubated in 10% (vol/vol) normal goat serum in PBS for 1 h at 22°C. After this step, the oocytes were incubated with 1 μg/ml of PSGL-1 peptide antibody or preimmune IgG for 1 h at 22°C. After washing, samples were incubated with 50 μg/ml of affinity-purified goat anti-rabbit IgG conjugated with HRP (Accurate Chemical Co., Westbury, NY) for 1 h at 22°C. The oocytes were rinsed and fixed with 2.5% glutaraldehyde (vol/vol) and 2% paraformaldehyde (wt/vol) in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.5 mM CaCl₂ at 4°C for 30 min. They were then processed for peroxidase cytochemistry and EM as previously described (Raub et al., 1994).

Sperm–Egg Binding Assay

A sperm–egg binding assay was set up according to a published procedure (Almeida et al., 1995). Porcine sperm cells and oocytes were gently washed once (1,000 rpm for 10 min) with HBSS/BSA (1% BSA in HBSS; wt/vol) except in controls where calcium and magnesium-free HBSS/BSA containing 2 mM EDTA was used throughout the entire assay. The sperm (0.1-ml aliquots of 2 × 10⁶ cells per ml) were mixed with oocytes (0.1-ml aliquots of ~500 oocytes per ml), in the presence or absence of 5 μM A23187, at 22°C for 1 h. The cell mixtures were carefully layered on the top of 100% dialyzed and heat-inactivated FCS (1 ml per tube) and spun at 500 rpm on a table-top centrifuge for 2 min (FCS “cushion”). Under these washing conditions, ~<10% of the free sperm cells were mechanically capacitated (Coons detected using a scanning electron microscope; Aarons et al., 1991), and more than 90% of the free sperm cells were viable according to their ability to exclude Hoechst 33258 (Tao et al., 1993). The supernatants were discarded and the cell pellets were fixed with 0.2 ml per tube of 2% paraformaldehyde (freshly prepared in PBS, wt/vol). The preparations were transferred onto glass microscope slides and examined under a microscope (Nikon Phase Contrast-2, EL WD 0.3; Tokyo, Japan), equipped with a screen monitor (VOCON Industries, Inc., New York) and a VCC200 video camera (VICON Industries, Inc., New York). The microscopic images were printed using a color video printer (UP-5200MD; Sony, Park Ridge, NJ).

For the inhibition studies, sperm cells were preincubated with mouse IgG, P23 mAb, or P7 mAb (all at 30 μg/ml) in the presence of 5 μM A23187 at 22°C for 30 min. Oocytes were preincubated with mouse IgM, CSLEX mAb, PLS mAb, human IgG, E-selectin Rg, or P-selectin Rg (all at 30 μg/ml) at 22°C for 30 min. The cells were then mixed with sperm cells without washing for the binding assay, as described above.

Sperm Cell–HL-60 Cell Binding Assay

Freshly collected sperm (0.3 ml) were resuspended into 30 ml of HBSS/BSA (1% heat-inactivated FCS in HBSS; vol/vol) and mixed with 2×10⁵ HL-60 cells (Ma et al., 1994; Asa et al., 1995). The labeled sperm cells as well as HL-60 and Ramos cells were washed three times with PBS (1,000 rpm for 10 min). The cells were resuspended in HBSS/BSA (2 × 10⁶ cells per ml), except in controls where calcium and magnesium-free HBSS/BSA containing 2 mM EDTA was used, throughout the assay. Under these washing conditions, ~30–50% of the sperm cells were mechanically capacitated as determined using Coomassie brilliant blue (Aarons et al., 1991), and >80% of the free sperm cells were viable based on their ability to exclude Hoechst 33258 (Tao et al., 1993).

The labeled sperm cells (1 × 10⁷ cells in a 0.5-ml aliquot) were mixed with either Ramos cells or HL-60 cells (2 × 10⁵ cells in a 0.1-ml aliquot) in the presence or absence of 5 μM A23187 at 22°C for 1 h, with end-to-end rotating. The unbound sperm cells were removed on a FCS cushion (0.5 ml of FCS per tube, centrifuged at 700 rpm on a table-top centrifuge for 2 min). The cell pellets were fixed with 2% paraformaldehyde (wt/vol; 0.5 ml per tube) for flow cytometric analysis (FACScan®). The binding of the fluorescence-labeled sperm cells to the Ramos or HL-60 cells was measured as the mean fluorescence intensity from >100,000 cells in the gated window for Ramos or HL-60 cells.

For the inhibition studies, the labeled sperm cells were preincubated with mouse IgG, P23 mAb, or P7 mAb (all at 30 μg/ml) in the presence of 5 μM A23187 at 22°C for 30 min. HL-60 cells were preincubated with either mouse IgM, CSLEX mAb, PLS mAb, or human IgG, E-selectin Rg, or P-selectin Rg (all at 30 μg/ml) at 22°C for 30 min. The cells were subsequently mixed with oocytes without washing for the binding assay.
Results

Expression of a P-selectin Ligand in Zona Pellucida of Porcine Oocytes

To investigate whether the zona pellucida of porcine oocytes contains a specific ligand for P-selectin, we took advantage of the cross-reactivity of human platelet P-selectin with porcine neutrophils, i.e., the fact that human P-selectin supports adhesion of porcine neutrophils (Geng, J.-G., unpublished observations). As shown in Fig. 1A, porcine zona pellucida contains many proteins as visualized by silver staining. Based on densitometric measurements, >50% of the total zona pellucida proteins were detected by silver staining. The silver-stained proteins were subsequently analyzed by P-selectin ligand blotting. As shown in Fig. 1B, P-selectin ligand blotting identified a dimeric molecule from porcine zona pellucida as a specific ligand for P-selectin. The binding of P-selectin to this molecule was calcium-dependent, as shown in Fig. 1C. In contrast, the binding of P-selectin to the zona pellucida proteins was not affected by the presence of 2 mM EDTA, as indicated. The blots were visualized with a chemiluminescence detection system (Amersham Corp.).

Figure 1. Ca²⁺-dependent binding of P-selectin to a dimeric molecule from porcine zona pellucida. (A) Silver staining of total zona pellucida proteins (~38 µg per lane) separated on 7% SDS-PAGE in the presence (R) or absence (N) of 5% β-mercaptoethanol (vol/vol). (B) P-selectin ligand blotting of zona pellucida proteins (~115 µg per lane), fractionated on 7% SDS-PAGE under nonreducing conditions. After electrophoresis, the proteins were transferred onto a blotting membrane and probed with human platelet P-selectin followed by a biotinylated P23 mAb in the presence of 1 mM CaCl₂ or 2 mM EDTA, as indicated. The blots were visualized with a chemiluminescence detection system (Amersham Corp.). (C) P-selectin ligand blotting of the zona pellucida proteins under reducing conditions. (D) P-selectin ligand blotting of the membrane extracts of porcine leukocytes (~0.5 mg per lane) under reducing (R) and nonreducing (N) conditions. Blotting procedures were exactly as described for B.

Figure 2. Immunoblotting of zona pellucida proteins with PSGL-1 peptide antibody. Porcine zona pellucida proteins, fractionated by 7% SDS-PAGE under nonreducing (A) and reducing (B and C) conditions and transferred to blotting membranes, were probed with rabbit preimmune IgG or PSGL-1 peptide antibody followed by biotinylated antibody to rabbit IgG. Immunoreactive proteins were visualized as outlined in the legend to Fig. 1.
of the proteins were recovered in the 40–70-kD ranges, under reducing conditions. This is consistent with published data (Dunbar et al., 1980).

In a parallel experiment, the zona pellucida proteins, separated by 7% SDS-PAGE and transferred to blotting membranes, were probed with human platelet P-selectin. Surprisingly, P-selectin specifically bound to a single protein with a molecular mass of ~210 kD under nonreducing conditions (Fig. 1 B, lane 1) and ~80 kD under reducing conditions (Fig. 1 C, lane 1). The binding was Ca\(^{2+}\) dependent as it was completely abolished by performing the P-selectin incubation in the presence of 2 mM EDTA (Fig. 1, B and C, lanes 2). P-selectin also specifically recognized a dimeric molecule from porcine leukocyte membrane extracts, with molecular masses identical to those of the zona pellucida protein under both nonreducing and reducing conditions (Fig. 1 D).

Three additional weaker bands (at ~210, ~180, and ~140 kD under reducing conditions) were also recognized by P-selectin (Fig. 1 C, lane 1). The presence of the ~210-kD band may be due to incomplete reduction of the ~210-kD dimer, a phenomenon frequently observed for human leukocyte PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994). The nature of ~180- and ~140-kD bands is unclear; they were not recognized by P-selectin (Fig. 1 B, lane 1) and PSGL-1 peptide antibody (see Fig. 3 A) when proteins were separated under nonreducing conditions. Plus, they were not recognized by PSGL-1 peptide antibody (Fig. 3, B and C), under reducing conditions. In addition, there was some background staining spots on the blots, which were not similar to the protein bands (Fig. 1 B, lane 2).

To corroborate the above findings, the porcine zona pellucida proteins were also probed with an antibody against a synthetic peptide encoding residues 41–55 of the amino acid sequence of PSGL-1. Fig. 2 shows that this antibody, but not preimmune IgG, bound to the ~210-kD protein under nonreducing conditions (A, arrow) and the ~80-kD protein under reducing conditions (B, arrow). Preincubation of the antibody with the synthetic peptide abrogated this binding (C, arrow). The protein bands at ~50–60 kD were most likely due to nonspecific binding, since (a) they existed in the blots probed with both preimmune IgG and PSGL-1 peptide antibody (A and B), and (b) they were not inhibited with the respective peptide antigen (C).

Using EM, the distribution of the P-selectin ligand in the zona pellucida of porcine oocytes was examined after labeling either with P-selectin followed by P-selectin antibody or with PSGL-1 peptide antibody. The experiments demonstrated that the P-selectin ligand was associated with membrane fragments and vesicles embedded throughout the zona pellucida at or near the (A) inner surface (arrows) of the zona pellucida, which is adjacent to the oocyte removed during the staining procedures, and (B) outer surface. (C) Zona pellucida stained in the absence of P-selectin. (D) Zona pellucida stained with P-selectin in the presence of EDTA. Bar, 500 nm.
the matrix of the zona pellucida (Figs. 3 and 4). The specificities of these approaches were confirmed by the absence, or marked reduction, of peroxidase reaction product in control oocytes, where either P-selectin (Fig. 3 C) or P-selectin antibody (data not shown) was omitted, where P-selectin was incubated in the presence of 2 mM EDTA (Fig. 3 D), or where preimmune IgG was used (Fig. 4 B).

**P-selectin Expression on Acrosomal Membrane of Porcine Sperm Cells**

The expression of P-selectin on porcine sperm cells was first established by FACS® analysis, using two different P-selectin antibodies, an FITC-conjugated rabbit P-selectin antibody and an FITC-conjugated P7 mAb. Both antibodies were raised against human platelet P-selectin and both reacted with porcine platelet P-selectin (see Fig. 7 C). As shown in Fig. 5, rabbit P-selectin antibody (A) and P7 mAb (B) bound to repeatedly washed sperm cells whose plasma membranes were no longer intact (see Fig. 8). By contrast, FITC-conjugated rabbit E-selectin antibody did not bind to the sperm cells (Fig. 5 C), although it clearly reacted with the TNF-α–treated PUVEC (D). Interestingly, P-selectin polyclonal antibody did not bind to the unwashed sperm cells (Fig. 6 A) unless they were treated with A23187 (a calcium ionophore known to induce the acrosomal reaction; Fig. 6 B), repeated washing (causing disruption of the plasma membranes, as demonstrated in Fig. 8; Fig. 6 C), or saponin (a detergent that selectively permeabilizes the plasma membrane; Fig. 6 D). Together, these results suggest that porcine sperm cells express P-selectin, but not E-selectin, and that P-selectin is expressed on the acrosomal membrane of the sperm cells, but not on the plasma membrane.

To corroborate the finding of P-selectin expression on porcine sperm cells, an immunoblotting experiment was carried out. Intact sperm cells were washed with ice-cold PBS and lysed in SDS sample buffer. The total sperm cell proteins were separated by SDS-PAGE and stained by silver staining. This resulted in numerous protein bands with various molecular masses, under reducing and nonreducing conditions (Fig. 7 A). The separated proteins were also transferred to blotting membranes and probed with either rabbit P- or E-selectin antibody or with P7 mAb. Both P-selectin antibodies bound to ~120-kD proteins under nonreducing conditions (Fig. 7 B, arrow). The observed molecular mass is identical to the molecular mass for human platelet P-selectin (Ma et al., 1994) and porcine platelet P-selectin (Fig. 7 C) (Toombs et al., 1995). The identities of the additional protein bands, with lower molecular masses, observed on the blots are not known. They may represent proteolytic fragments of P-selectin generated.
during the lysis of the sperm cells, since (a) sperm cells were known to contain a variety of proteases (Eddy, 1988), and (b) the antibodies used in this experiment specifically bound to P-selectin among all the proteins present in the platelet lysates (Fig. 7 C). The relatively sharper ~120-kD bands from sperm cells (Fig. 7 B, arrow), as compared with the relatively broad bands from platelets (Fig. 7 C), could also result from the proteolytic cleavage of some forms of the sperm P-selectin. Differential protease accessibility of heterogeneous glycosylated platelet P-selectin has been demonstrated previously (Johnston et al., 1989). E-selectin antibody did not recognize any protein in the porcine sperm cells, although it bound avidly to a ~90-kD protein from TNF-α–treated PUVEC (Fig. 7 D).

Further studies, using immunoelectron microscopy and a gold-conjugated rabbit P-selectin antibody, revealed that the localization of P-selectin on the porcine sperm cells was confined to the region of the sperm head, containing the dense nucleus, covered only by the exposed acrosome (Fig. 8 A, 1), including the acrosomal cap (Fig. 8 A, arrow). The gold-conjugated P-selectin antibody did not label the lower one-third of the sperm head (Fig. 8 A, 2), the neck (Fig. 8 A, 3), or the tail (Fig. 8 A, 4). Consistent with the results from the flow cytometric studies (Fig. 6), label was not observed on sperm cells with the plasma membrane still intact (Fig. 8 B). Incubation of sperm cells with the gold-conjugated P-selectin antibody in the presence of the unconjugated P-selectin antibody abolished the binding (Fig. 8 C). Again, gold-conjugated rabbit E-selectin antibody did not label the sperm cells (Fig. 8 D). Neither the broken plasma membrane (Fig. 8 D, arrow) nor the acrosome was labeled, although it labeled the surface of a CHO cell line expressing human E-selectin (Fig. 8 E). Thus, these results confirm the expression of P-selectin, but not E-selectin, on the acrosomal membranes, but not on the plasma membrane, of porcine sperm cells. Furthermore, the detection of P-selectin after acrosomal reaction suggests the possible localization of the molecule on the inner acrosomal membrane.
Function of the Oocyte P-selectin Ligand and the Sperm Cell P-selectin

To evaluate the function of the P-selectin ligand in the zona pellucida of porcine oocytes and P-selectin on the acrosomal membrane of porcine sperm cells, an in vitro sperm–oocyte binding assay was carried out, essentially according to the published procedure (Almeida et al., 1995). In this assay, acrosome-reacted sperm cells bound to oocytes in numbers usually exceeding 15 sperm cells per oocyte (referring to the number of bound sperm cells viewed in the single optical plane used; the total number of sperm cells bound to the entire oocyte was considerably larger; Table I). The binding was Ca\(^{2+}\) and Mg\(^{2+}\) dependent, since it could be dramatically reduced (to less than three sperm cells per oocyte) by treatment with EDTA. The requirement for divalent cations for sperm–egg binding is consistent with previous observations (Yanagimachi, 1988).

Sperm–egg binding was also significantly reduced (to less than five sperm cells per oocyte) by preincubation of the sperm cells with P7 (a leukocyte adhesion blocking mAb against P-selectin), or by preincubation of the oocytes with PL5 (a leukocyte adhesion blocking mAb against PSGL-1), purified platelet P-selectin, or recombinant P-selectin Rg (Table I). By contrast, the sperm–egg binding was not affected by preincubation of the oocytes with mouse IgM, CSLEX (an mAb against SLex), mouse IgG, P23 (a leukocyte adhesion nonblocking mAb against P-selectin), or E-selectin Rg (Table I).

These results strongly suggest that both the P-selectin ligand in the zona pellucida of porcine oocytes and P-selectin on the acrosome-reacted sperm cells are biologically func-
Table I. Adhesion of Sperm Cells to Oocytes

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<th>Condition</th>
<th>Sperm cells per oocyte*</th>
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<tbody>
<tr>
<td>EDTA</td>
<td>18 ± 3</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>P23 mAb</td>
<td>19 ± 5</td>
</tr>
<tr>
<td>P7 mAb</td>
<td>4 ± 3</td>
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<td>Mouse IgM</td>
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<tr>
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<td>2 ± 2</td>
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<tr>
<td>P-selectin</td>
<td>16 ± 5</td>
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Porcine sperm cells (2 × 10⁶ cells per ml) and oocytes (~500 oocytes per ml) were resuspended in HBSS/BSA (–), or calcium and magnesium–free HBSS containing 2 mM EDTA (EDTA). The sperm cells and oocytes were mixed in the presence of A23187 unless specifically indicated. Aliquots of oocytes were preincubated separately with mouse IgM, CSLEX (an mAb against SLex), PL5 (an mAb against PSGL-1; all at 30 μg/ml), or platelet P-selectin (10 μg/ml), or human IgG, P-, and E-selectin Rgs (all at 30 μg/ml), respectively. Aliquots of sperm cells were preincubated with mouse IgG, P7 (a leukocyte adhesion blocking mAb against P-selectin), or P23 (a leukocyte adhesion nonblocking mAb against P-selectin; all at 30 μg/ml) at 22°C for 30 min. All results were expressed as the mean ± SD number of adherent sperm cells per oocyte in the optical plane studied in five separate experiments; more than 50 oocytes were observed for each condition.

*Refers to the number of bound sperm cells viewed in the single optical plane used.

To corroborate this finding, we investigated whether porcine sperm cells could bind to HL-60 cells, a human promyeloid cell line that expresses the functional PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995; Spertini et al., 1996; Tu et al., 1996). Fig. 9 shows that, in the presence of Ca²⁺, Mg²⁺, and A23187, fluorescently labeled sperm cells avidly bound to HL-60 cells (A4), but not to Ramos cells (A1 and A2), a human lymphoblast cell line that does not express the functional PSGL-1 (Vachino et al., 1995). This binding was reduced when the experiment was carried out in the absence of A23187 (A3), a calcium ionophore that induces the sperm acrosomal reaction (Aarons et al., 1991; Tao et al., 1993). The partial binding observed in the absence of A23187 (A3) was likely attributed to the broken cytoplasmic membrane on some sperm cells, caused by the repeated washing procedures used in their preparation (Fig. 8). The requirement for A23187 for full binding activity is consistent with the expression of P-selectin on the acrosomal membrane of sperm cells (Figs. 6 and 8).

As expected, the binding activity was blocked by preincubation of the sperm cells with P7 (a leukocyte adhesion blocking mAb against P-selectin; B3), but not with mouse IgG (B1) or P23 (a leukocyte adhesion nonblocking mAb against P-selectin; B2). Preincubation of HL-60 cells with PL5 (a leukocyte adhesion blocking mAb against PSGL-1; C3) or P-selectin Rg (D3) also neutralized the binding, but mouse IgM (C1), human IgG (D1), or E-selectin Rg (D2) did not. Taken together, the results provide independent and convergent evidence for the biological function of the zona pellucida P-selectin ligand and the sperm P-selectin.

It should be mentioned that the anti-SLex mAb, CSLEX, partially inhibited the interaction in this assay. This partial inhibition, by CSLEX mAb, of the binding of sperm cells to HL-60 cells (Fig. 9; C2), but not on the binding of sperm cells to the oocytes (Table I), may be due to subtle differences in assay formats. Alternatively, the difference in cell type (different oligosaccharide structures on porcine oocytes vs human HL-60 cells) and/or the particulars of the CSLEX mAb specificity (recognizes other carbohydrate epitopes besides SLex; Stroud et al., 1996a,b) may contribute to this partial inhibition. In addition, the lack of inhibition by E-selectin Rg, particularly in sperm cell binding to HL-60 cells (Fig. 9; D2), raises several possibilities, such as (a) there is an insufficient amount of E-selectin Rg in the assay; (b) the binding determinant(s) on PSGL-1 for P- and E-selectin is quite distinct and separated; (c) there is sufficient other ligand(s) for E-selectin; or (d) PSGL-1 is not a functional ligand for E-selectin. Obviously, further experiments are required to clarify these issues.

Discussion

Attachment of the sperm cell to the oocyte is the first step in mammalian fertilization. This process involves a cascade of cell–cell and cell–matrix interactions with at least...
The specific distribution pattern of the P-selectin ligand in the zona pellucida matrix argues against the possibility that the detection of the ligand in this tissue represents an artifact. An erroneous signal caused by contaminating leukocytes is unlikely for the following reasons. First, as discussed above, our microscopic studies show that both P-selectin and PSGL-1 peptide antibody specifically bind to membranous structures within the zona pellucida. Second, large amounts of leukocytes (~0.5 mg proteins of membrane extract from ~100 million leukocytes per lane) are typically required for positive detection with the P-selectin blotting method used in this report (Fig. 1D; Ma et al., 1994). Hence, if the molecule detected in the zona pellucida represents the leukocyte ligand, truly major leukocyte contaminations of the zona pellucida preparations would be required to produce the signal observed on the blots. However, light and EM examination of these preparations failed to show such leukocyte presence. Third, nonspecific binding of P-selectin and PSGL-1 peptide antibody to a contaminating or otherwise unrelated polysaccharide and/or protein structure(s) on the surface of the membranous structures in the zona pellucida is unlikely since our blotting experiments clearly show that both P-selectin and PSGL-1 peptide antibody primarily recognize one protein among the considerable numbers of proteins present in the zona preparation; in addition, this protein is one of the minor constituents of the zona pellucida (Fig. 1, compare A, B, and C). Finally, the expression of a P-selectin ligand in the zona pellucida is consistent with the detection of PSGL-1 mRNA in the mouse ovary, as demonstrated by Northern analysis (Yang et al., 1996).

The P-selectin ligand from porcine zona pellucida and leukocytes described in this report shares many characteristics with human leukocyte PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995). These include biochemical properties (disulfide-linked dimeric protein), functional properties (recognition by P-selectin in a Ca\(^{2+}\)-dependent manner), and polypeptide properties (recognition by PSGL-1 peptide antibody). However, there is a difference in molecular masses between the human leukocyte PSGL-1 (~240 kD under nonreducing conditions and ~100 kD under reducing conditions) (Ma et al., 1994) and the porcine oocyte and leukocyte P-selectin ligands (~210 kD under nonreducing conditions and ~80 kD under reducing conditions) (Figs. 1 and 2). Therefore, since amino acid sequencing information is not available for the porcine oocyte P-selectin ligand, the question as to whether this molecule is identical to PSGL-1 remains to be answered.

In this study we failed to detect any expression of E-selectin on sperm cells by flow cytometry, immunoblotting, or immunogold EM. The apparent absence of the expression of E-selectin on porcine sperm cells argues against a biological role for this lectin in gamete interactions. However, an immunohistologic study has demonstrated the expression of both E- and P-selectin on vascular endothelial cells in the decidua basalis, but not on decidua parietalis (Burrrows et al., 1994). Therefore, it is conceivable that E-selectin, along with P-selectin, on vascular endothelial cells in the decidua basalis may interact with the zona pellucida ligand during trophoblast implantation.

The expression of P-selectin on the acrosomal membrane of porcine sperm cells is supported by several experimental findings. First, blotting of sperm cell extracts separated on SDS-PAGE with two well-characterized P-selectin antibodies (Ma et al., 1994; Asa et al., 1995; Toombs et al., 1995) resulted, for both reagents, in the detection of a protein band with an apparent molecular mass identical to those of human platelet P-selectin (Ma et al., 1994) and porcine platelet P-selectin (Fig. 7). Since purified sperm cells (see Materials and Methods) were used for this experiment, it is unlikely that platelet contamination is responsible for the signal on the blot; as argued above, a considerable amount of platelets are required for a positive signal in this type of experiment. Second, immunoelectron microscopies of the porcine sperm cells quite unequivocally demonstrate the abundant presence of a molecule recognized by a P-selectin antibody, on what appears to be the acrosomal membrane of the sperm cells (Fig. 8). This distribution to a specific membranous compartment on the sperm cells is in itself an argument against nonspecific labeling, caused by unspecific binding of the antibody as well as by unspecific adsorption of (soluble) antigen to the sperm cell surface. The fact that only acrosome-reacted sperm cells are capable of binding the antibodies also argues against nonspecific results. Finally, the capacity of acrosome-reacted porcine sperm cells to attach to oocytes and HL-60 cells in a manner that is inhabitable by P-selectin antibodies strongly suggests a P-selectin function on the acrosome-reacted sperm cells (Table I; Fig. 9).

The ultrastructural distribution of the zona pellucida P-selectin ligand indicates that it is less likely a component of the zona pellucida matrix. Instead, the molecule appears to be located exclusively in membranes embedded within the matrix of the zona pellucida. The membrane structures in the zona pellucida may originate from the long oocyte microvilli and/or from the follicle cell projections that transverse the zona pellucida and make contact (gap junctions) with the oocyte plasma membrane (Austin, 1968). The morphological localization of P-selectin ligand-containing membranes in the zona pellucida implicates a potentially functional role for a P-selectin ligand during...
the penetration by the acrosome-reacted sperm cells through the thick, gel-like matrix of the zona pellucida. In this regard, the expression of functional P-selectin on the acrosomal membrane of sperm cells supports this hypothesis.

The findings in this report implicate a potentially biological role for the zona pellucida P-selectin ligand and the sperm P-selectin in porcine gamete interactions. However, since homozygous mice deficient in P-,- or L-selectin, by homologous recombination, have no apparent deficiencies in breeding (Myadas et al., 1993; Arbonès et al., 1994; Labow et al., 1994), this interaction may be only one of several molecular mechanisms involved in fertilization in vivo (Wassarman, 1995; Snell and White, 1996). A similar, apparently redundant pathway has been described for leukocyte-endothelial cell interactions. The phenotypes of P-, E-, and L-selectin knockout mice appear normal until the animals are challenged by inflammatory mediators (Myadas et al., 1993; Arbonès et al., 1994; Labow et al., 1994).

Taken together, our in vitro studies suggest that a mechanism, similar to that involved in leukocyte recruitment, may be involved in sperm-egg binding. However, the specific role(s) of these molecules in the different steps of sperm-egg interaction, especially in vivo, require further investigation.

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References


