Acrosome Reaction of Mouse Epididymal Sperm on Oocyte Zona Pellucida

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Abstract. To improve assessment of the acrosome reaction of mouse epididymal sperm, we employed anti-Izumo1 antibody instead of antibodies against acrosomal proteins. The acrosomal states among acrosome-intact, spontaneously acrosome-reacted, truly acrosome-reacted, and probably dead and/or membrane-damaged sperm were clearly distinguished by combined application of anti-Izumo1 antibody, DNA dye Hoechst 33342, and monoclonal antibody MN7 to paraformaldehyde-fixed sperm. When the acrosome reaction of capacitated epididymal sperm on the oocyte zona pellucida was examined using anti-Izumo1 antibody, approximately 20% of sperm bound onto the zona pellucida were acrosome-reacted 30 min after insemination. We also observed the moment of the acrosome reaction of live sperm on the zona pellucida by time-lapse monitoring using fluorescein isothiocyanate-conjugated anti-Izumo1 antibody.

Key words: Acrosome reaction, Izumo1, Oocyte, Sperm, Zona pellucida

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Mammalian sperm possess an exocytotic vesicle, acrosome, in the apical region of anterior head overlying the nucleus [1–3]. Following the binding of acrosome-intact sperm to the zona pellucida (ZP), an extracellular matrix of surrounding the oocyte, the sperm undergo the acrosome reaction, which is a fusion event between the outer acrosomal and plasma membranes. Consequently, the acrosomal components of sperm are released, and then acrosome-reacted or possibly acrosome-reacting sperm penetrate the ZP. The acrosome reaction is physiologically essential because only acrosome-reacted sperm are capable of fusing with the oocyte plasma membrane [1, 4, 5]. Thus, the ZP mediates the acrosome reaction of fertilizing sperm on the ZP surface. However, most studies concerning the acrosome reaction deal with a model system using the soluble form of ZP.

The acrosomal status of sperm is assessed by several analytical methods, including transmission electron microscopy [6, 7] and cytochemical staining using Coomassie Brilliant Blue (CBB) [8], chlortetracline [9–11], plant lectins [12], or antibodies against acrosomal proteins [13–15]. Electron microscopic observation directly reflects the acrosomal status, but a special research facility is required for this analysis [1]. Sperm staining assays have been widely utilized due to the relatively easy techniques. In the case of the above protein dye and antibodies, the acrosomal status can be evaluated on the basis of the presence (or absence) of acrosomal matrix proteins. One of the disadvantages of these assays is that dead and moribund sperm are detected together with live acrosome-intact sperm. It is also difficult to assess...
the acrosome reaction of mouse and rat sperm, since the acrosome is very thin and tightly attached to the sperm head [1, 2].

In this study, we examined the acrosome reaction of mouse sperm on the surface of the ZP in vitro. Instead of antibodies against acrosomal proteins, antibody against Izumo1, which is involved in sperm/oocyte fusion [16], was used to assess the acrosomal status. The reasons for use of this antibody were that (1) Izumo1 is entirely absent on the cell surface of fresh live sperm, (2) Izumo1 is localized on the sperm head only after the acrosome reaction, and (3) Izumo1 is only present in the acrosomal cap region when sperm are probably dead and/or membrane-damaged [16–18]. Immunocytochemical analysis using anti-Izumo1 antibody indicates that approximately 20% of ZP-bound sperm undergo the acrosome reaction 30 min after insemination. We also show time-lapse observation of the acrosome reaction of live sperm on the ZP surface.

Materials and Methods

Materials

Calcium ionophore A23187 and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alexa Flour 488 and 568-conjugated goat antibodies against rabbit IgG and mouse IgG, respectively, were purchased from Invitrogen (Eugene, OR, USA). Monoclonal antibody MN7 [19] was the generous gift of Dr. K. Toshimori. Polyclonal anti-Izumo1 antibody [16], fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody OBF13 (anti-Izumo1 antibody) [17], and transgenic mice [C57BL/6 Tg(CAG/Acr-EGFP)C3-N01-FJ002Os] expressing a transgene, Acr3-enhanced green fluorescent protein (EGFP), containing three DNA fragments encoding the promoter region of mouse acrosin, EGFP derived from jellyfish GFP, and bovine growth hormone polyadenylation signal [20] were the gifts of Dr. M. Okabe. ICR and BDF1 mice were obtained from Japan SLC (Shizuoka, Japan). All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals in University of Tsukuba.

Preparation of sperm samples

Fresh cauda epididymal sperm from ICR mice (3–6 months old) were dispersed in a 0.2-ml drop of TYH medium [21] containing glucose, sodium pyruvate, bovine albumin, and antibiotics at 37 °C under 5% CO2 in air for 10 min, and were capacitated by incubation at 37 °C under 5% CO2 in air for 60 min. Capacitated sperm were induced to undergo acrosome reaction by addition of calcium ionophore A23187 at a final concentration of 5 µg/ml followed by incubation at 37 °C under 5% CO2 in air for 60 min, as described previously [22]. The sperm samples were transferred into a 1.5-ml microtube, washed with phosphate-buffered saline (PBS) by centrifugation at 3,000 rpm for 5 min, fixed in PBS containing 4% paraformaldehyde on ice for 15 min, and then washed with cold PBS. For permeabilization, paraformaldehyde-fixed sperm were further treated with 0.1% Triton X-100 in PBS on ice for 15 min.

Cytocchemical staining of sperm

Fixed sperm were blocked in PBS containing 3% normal goat serum and 0.05% Tween-20 (PBS/NGS/T20) on ice for 30 min, washed with PBS/NGS/T20 by centrifugation, incubated with anti-Izumo1 antibody and monoclonal antibody MN7 in PBS/NGS/T20 for 60 min, washed, and then reacted with Alexa Flour 488-conjugated anti-rabbit IgG and Alexa Flour 568-conjugated anti-mouse IgG antibodies in PBS/NGS/T20 for 60 min. After washing with PBS, sperm cells were stained with Hoechst 33342 (2.5 µg/ml) for 30 min, mounted, and then observed under an IX-71 fluorescent microscope (Olympus, Tokyo, Japan) equipped with a SPOT RT SE18 camera (Diagnosis Instruments, Sterling Heights, MI, USA), as described previously [23]. Fixed sperm were also stained in a 0.04% CBB solution at room temperature for 5 min, washed with PBS, and viewed under an Olympus IX-71 microscope.

Acrosome reaction on oocyte ZP

BDF1 mice (7–10 weeks old) were superovulated by intraperitoneal injection of pregnant mare’s serum gonadotropin (5 units; Teikoku Zoki, Tokyo, Japan) followed by human chorionic gonadotropin (5 units; Teikoku Zoki) 48 h later. Cumulus mass containing metaphase II-arrested oocytes was collected from the oviductal ampulla of the superovulated mice 14 h after injection of human...
chorionic gonadotropin, placed in a 0.1-ml drop of TYH medium covered with mineral oil, treated with bovine testicular hyaluronidase (350 units/ml; Sigma-Aldrich), and washed with TYH medium, as described previously [24]. Fresh cauda epididymal sperm were capacitated by incubation in a 0.2-ml drop of TYH medium at 37°C under 5% CO₂ in air for 60 min. An aliquot (3 × 10⁴ or 5 × 10⁴ sperm/10 µl) of the capacitated sperm suspension was mixed with the cumulus-free oocytes in a 90-µl drop of TYH medium, and the mixture was incubated at 37°C under 5% CO₂ in air for 30 min. The oocytes were transferred to a 0.1-ml drop of fresh TYH medium, washed by rotary shaking of the drops on Petri dish to remove loosely bound and unbound sperm, and fixed in PBS containing 4% paraformaldehyde on ice for 15 min. After washing with PBS containing 0.5% polyvinylpyrrolidone, sperm tightly bound onto the ZP were immunostained with anti-Izumo1 antibody, and observed under an IX-71 fluorescence microscope, as described above.

Time-lapse fluorescence imaging of sperm
Cumulus-free oocytes were inseminated with capacitated epididymal sperm (3 × 10⁴ sperm/10 µl) in TYH medium, and the mixture was immediately transferred to a drop of fresh TYH medium containing FITC-conjugated anti-Izumo1 monoclonal antibody on a 3.5-cm glass-bottomed dish (Matsunami Glass, Osaka, Japan), and observed at 5-sec intervals under the above IX-71 fluorescence microscope, as described above [25]. Images were acquired and processed using a MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA).

Results and Discussion
We first carried out immunocytochemical analysis of cauda epididymal sperm before and after calcium ionophore A23187-induced acrosome reaction, using anti-Izumo1 antibody (Fig. 1). Monoclonal antibody MN7, which specifically recognizes a 90-kDa intra-acrosomal protein of mouse, rat, and hamster sperm [19], was used as a control. Antibody against transmembranous protein Izumo1 gave no immunostaining signal on live sperm, and only recognized the protein on the sperm head following the acrosome reaction (H-type staining pattern, see Fig. 1A), as describe previously [16–18]. Izumo1 was also present in the acrosomal cap region of presumably dead and/or membrane-damaged sperm (A-type staining pattern). We also observed spontaneously acrosome-reacted and/or acrosome-reacting sperm still containing the MN7 antigen, probably owing to incomplete dispersal from the acrosome matrix (Fig. 1B). In these sperm, Izumo1 was already spread or spreading on the sperm head. It is therefore possible that the spread of Izumo1 may occur immediately after fusion between the outer acrosomal and plasma membranes of sperm, even if some of the acrosomal proteins remain in the matrix.

The Izumo1 and MN7 antigens were both undetected in the head and acrosome regions of fresh sperm fixed with paraformaldehyde, respectively (Fig. 1C), although H-type sperm most likely corresponding to spontaneously acrosome-reacted sperm were occasionally immunostained with anti-Izumo1 antibody (arrows). Following successive treatment of paraformaldehyde-fixed sperm with Triton X-100, the Izumo1 and MN7 antigens were detected in the acrosomal cap region (panels j, k, and l). In acrosome-reacted sperm induced by calcium ionophore A23187, anti-Izumo1 antibody gave the H-type staining pattern (panel n), as described above. No significant signal was recognized by monoclonal antibody MN7, but some sperm still contained MN7 antigen in the acrosomal matrix due to incomplete dispersal (arrowheads in panels o and p). These data verify that application of anti-Izumo1 antibody to paraformaldehyde-fixed sperm without Triton X-100 treatment enables acrosome-reacted sperm to be distinguished from acrosome-intact sperm, as described previously [16–18]. However, we could not distinguish between truly and spontaneously acrosome-reacted sperm because anti-Izumo1 antibody recognizes both types of acrosome-reacted sperm.

When the acrosomal status of epididymal sperm was assessed by CBB staining, 86% of sperm were found to have lost the stained signal from the acrosome after treatment with calcium ionophore A23187 (Fig. 2A). Anti-Izumo1 antibody gave the H-type staining pattern to 12, 15, and 85% of sperm before and after 60-min incubation, and after the A23187 treatment, respectively. Thus, approximately 70% of sperm were acrosome-reacted by calcium ionophore A23187 under the
conditions employed in this study. The proportions of A-type sperm immunostained with anti-Izumo1 antibody were similar to those of sperm immunostained with monoclonal antibody MN7 (Fig. 2B). The MN7 antigen-bearing sperm fixed only with paraformaldehyde as well as the A-type sperm may have been dead, moribund, and/or membrane-damaged. In some experiments, we examined whether EGFP fluorescence in the acrosome is influenced by freeze-thaw treatment of EGFP-expressing transgenic mouse sperm. EGFP fluorescence completely disappeared likely due to the loss of the acrosome and the damage to the sperm membranes [1], although the freeze-thawed sperm still exhibited the A-type staining pattern using anti-Izumo1 antibody (data not shown), as described previously [18].

We next examined the acrosomal status of epididymal sperm bound on the ZP of metaphase II-arrested oocytes in vitro (Fig. 3A). The cumulus-free, ZP-intact oocytes were inseminated with capacitated epididymal sperm, incubated for 30 min, washed, and then fixed with paraformaldehyde. Spontaneously acrosome-reacted and dead/membrane-damaged sperm were eliminated by the washing step because at least in the mouse, sperm that have spontaneously undergone the acrosome reaction are incapable of binding to the ZP [1, 26, 27]. Indeed, spontaneously acrosome-reacted sperm that had been previously labeled with FITC-conjugated anti-Izumo1 monoclonal antibody barely bound onto the ZP (data not shown). When capacitated sperm (3 × 10⁴ and 5 × 10⁴ cells/0.1 ml) were mixed with the oocytes, approximately 30 and 50 sperm bound onto the ZP (mean ± SD=31.1 ± 7.4 and 51.5 ± 16.9 sperm/oocyte for 10 and 31 oocytes), and 20 and 24% of ZP-bound sperm (19.7 ± 6.1% and 24.2 ± 8.7%, respectively) exhibited the H-type staining pattern using anti-Izumo1 antibody. We also carried out time-lapse monitoring of the acrosome reaction of live sperm on the ZP using EGFP-expressing transgenic mouse sperm and FITC-conjugated anti-Izumo1 antibody (Fig. 3C). The
EGFP fluorescence of a ZP-binding sperm disappeared from the acrosome within 10 sec, while the FITC-conjugated antibody labeled the entire region of a sperm head at least by 5 sec. These results emphasize the usefulness of anti-Izumo1 antibody for evaluation of the acrosome reaction of sperm on the ZP. It is important to note that we could not observe penetration of the above two acrosome-reacted sperm into the ZP.

The acrosomal status of sperm is usually...
assessed by sperm staining assays using CBB or antibodies against acrosomal proteins [1]. Since assessment of the acrosome reaction is based on disappearance of the stained signal from the acrosome, there are some difficulties in judging the complete loss of acrosomal proteins. Indeed, some acrosomal matrix proteins still remain associated with acrosome-reacted sperm (Figs. 1 and 2), as described previously [2, 28]. In contrast, the staining assay using anti-Izumo1 antibody has several advantages over assays using antibodies against acrosomal proteins, including the absence of staining signal in acrosome-intact sperm, and the presence of the widespread signal pattern over the entire sperm head region. Although it is important to examine the acrosome reaction on the ZP, solubilized ZP is widely used for understanding the exocytotic event. Thus, the anti-Izumo1 antibody-based experimental procedure presented in this study will be helpful in further elucidating the molecular mechanism of the acrosome reaction on the ZP.

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**References**


