Hyaluronan-Degrading Activity of Mouse Sperm Hyaluronidase Is Not Required for Fertilization?

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Abstract. Sperm hyaluronidase has long been believed to participate in sperm penetration through the cumulus matrix. However, our previous works using male mice lacking either one of two sperm hyaluronidases, SPAM1 and HYAL5, conclusively showed that neither of these hyaluronidases is essential for fertilization. In this study, we examined whether the hyaluronan-degrading activity of mouse epididymal sperm is indeed required for the fertilization process. When the oocyte-cumulus complex was incubated with sperm protein extracts or capacitated epididymal sperm in the presence of the hyaluronidase inhibitor apigenin, dispersal of cumulus cells from the cumulus was effectively inhibited. Despite the presence of apigenin, capacitated epididymal sperm normally entered the oocyte-cumulus complex, traversed the cumulus matrix and reached the oocyte zona pellucida. Importantly, epididymal sperm were also capable of normally fertilizing the metaphase II-arrested oocytes in the presence of apigenin. These data suggest that the hyaluronan-degrading activity of sperm hyaluronidase may not be required for fertilization, at least in the mouse.

Key words: Cumulus Hyaluronidase, Mouse, Oocyte, Sperm

Mammalian fertilization requires sperm to enter the oocyte-cumulus complex (OCC) and penetrate the cumulus matrix surrounding the oocytes [1–5]. Cumulus cells are embedded in the extracellular matrix containing hyaluronan (hyaluronic acid or hyaluronate), a polymer consisting of a repeating disaccharide unit of N-acetyl-D-glucosamine and D-glucuronic acid [6]. Consequently, sperm hyaluronidase has long been believed to catalyze degradation of hyaluronan in the cumulus matrix, thus enabling the sperm to reach the oocyte zona pellucida (ZP) [1, 7]. The cumulus matrix is also known to participate in stimulating sperm motility and/or in promoting the acrosome reaction, in addition to control of sperm access to the oocyte ZP [1, 7]. Indeed, the fertilization rate is extremely reduced by removal of the cumulus matrix in vitro [1]. Although the cumulus beneficially affects fertilization, the molecular mechanism of sperm entry into the cumulus and subsequent penetration still remains unclear.

Mouse epididymal sperm contain at least two hyaluronidases, SPAM1 and HYAL5, glycosylphosphatidylinositol (GPI)-anchored on the plasma and/or acrosomal membranes [8–11]. We previously demonstrated that SPAM1 is localized only on the plasma membrane of sperm and that acrosome-reacted sperm still retain approximately half of SPAM1 [9]. HYAL5, on the other hand, is present on the plasma and acrosomal membranes of acrosome-intact sperm and is mostly released from the sperm membranes during the acrosome reaction [9, 10]. On the basis of the subcellular distribution, both HYAL5 and SPAM1 on the plasma membrane may be involved in sperm penetration through the cumulus matrix, and acrosomal HYAL5 released by the acrosome reaction may play an important role in the local hyaluronan hydrolysis near or on the ZP surface to enable the proximal region of the sperm tail to move freely [10]. Inconsistent with these possibilities, male mice lacking either one of these two hyaluronidases (Spam1–/– or Hyal5–/–) have been shown to be fully fertile [9, 11], providing evidence that neither SPAM1 nor HYAL5 is essential for fertilization in the mouse. However, in vitro fertilization (IVF) assays have revealed that Spam1–/– sperm are distinguished from wild-type and Hyal5–/– sperm by significant delays in entering the OCC, dispersing cumulus cells from the OCC and traversing the cumulus matrix to reach the ZP [11]. Thus, only SPAM1 may play a particular role in sperm accessibility to the oocyte ZP.

Our previous works concerning Spam1–/– and Hyal5–/– mice [9–11] raise a fundamental question of whether sperm hyaluronidase is a prerequisite for the fertilization processes including sperm penetration through the cumulus matrix. In this study, to directly address the question, we carried out IVF assays of mouse epididymal sperm in the presence of a hyaluronidase inhibitor, apigenin [12–14].

Materials and Methods

Animal experiments

All animal experiments were carried out ethically according to the Guide for the Care and Use of Laboratory Animals at University of Tsukuba.

Preparation of sperm extracts

Fresh cauda epididymal sperm of ICR mice (3–5 months old; Japan SLC, Shizuoka, Japan) were collected in TYH medium [15], washed with phosphate-buffered saline (PBS) by centrifugation at 800 × g for 10 min and extracted in 20 mM Tris/HCl, pH 7.4, containing 1% Triton X-100, 0.15 M NaCl and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) on ice for 6 h. The

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sperm suspension was centrifuged at 13,000 x g for 10 min, and the supernatant solution was used as “sperm protein extract,” as described previously [11].

**Measurement of enzyme activity**

Hyaluronidase activity was measured by the colorimetric method using Alcian Blue 8GX (Sigma-Aldrich), as described previously [9]. Briefly, the reaction mixture (0.3 ml) consisted of 50 mM sodium acetate, pH 6.0, 50 mM MgCl₂, 20 μg rooster comb hyaluronan (Wako, Osaka, Japan) and sperm protein extracts (10 μg). Following incubation at 37 °C for 30 min, the reaction was terminated by addition of 0.02% Alcian Blue solution (0.3 ml), and the mixture was centrifuged at 13,000 x g for 5 min. Absorbance of the supernatant solution at 603 nm was measured using a Shimadzu UV-160 spectrophotometer (Kyoto, Japan). A standard curve of hyaluronidase activity was obtained using bovine testicular hyaluronidase (295 units/mg, Sigma-Aldrich H3506) under the above conditions. The protein concentration was determined using a Bio-Rad protein assay reagent kit (Thermo Fisher Scientific, Rockford, IL, USA).

**Hyaluronan zymography**

Proteins exhibiting hyaluronidase activity were visualized by SDS-PAGE in the presence of 0.01% rooster comb hyaluronan under nonreducing conditions, as described previously [9]. After electrophoresis, gels were washed with 50 mM sodium acetate buffer, pH 6.0, containing 0.15 M NaCl and 3% Triton X-100 at room temperature for 2 h to remove SDS and then incubated in the same buffer free of Triton X-100 at 37 °C overnight. The hyaluronan-degrading proteins were detected as transparent bands against a blue background by staining the gels with 0.5% Alcian Blue 8GX and 0.25% Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

**Dispersal of cumulus cells**

Female ICR mice (8–10 weeks old; Japan SLC) were superovulated by intraperitoneal injection of pregnant mare’s serum gonadotropin (Serotropin, 5 units; Askia Pharmaceutical, Tokyo, Japan) followed by human chorionic gonadotropin (Gonatropin 3000, 5 units; Askia Pharmaceutical) 48 h later. The OCCs containing metaphase II-arrested oocytes were collected from the oviductal ampulla 14 h after injection of human chorionic gonadotropin and placed in a 90-μl drop of TYH medium containing bovine serum albumin (4 mg/ml, Sigma-Aldrich, A3311-10G) and various concentrations of apigenin (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) as a solvent. Fresh cauda epididymal sperm from ICR mice (3–5 months old) were capacitated by incubation for 2 h in a 0.2-ml drop of TYH medium at 37 °C under 5% CO₂ in air. An aliquot (10 μl) of the capacitated sperm suspension (1.5 x 10⁷ cells/μl) was mixed with the OCCs in TYH medium containing 0.05 mM by approximately 50%, whereas a very low level of activity (less than 4% of the total activity in the absence of apigenin) was found at 0.5 and 1 mM apigenin (Fig. 1A). Hyaluronan zymography indicated the presence of two hyaluronan-degrading proteins corresponding to 55-kDa HYAL5 and 52-kDa SPAM1 in the protein extracts (Fig. 1B). As expected, the hyaluro-
nan-degrading activities of HYAL5 and SPAM1 were not detectable when the gels were incubated in the presence of 0.5 and 1 mM apigenin. Thus, consistent with previous works [12–14], apigenin efficiently inhibited the hyaluronan-degrading activity of sperm hyaluronidase.

When the effect of apigenin on dispersal of cumulus cells from the OCC was examined, cumulus cells were readily dispersed from the OCC by sperm protein extracts in the absence of apigenin (Fig. 2). The oocytes completely lost cumulus cells 90 min after addition of the sperm extracts. The levels of cumulus cell dispersal decreased with increasing concentrations of apigenin (0.1, 0.5 and 1 mM). In addition, cumulus cells were barely dispersed in the presence of 1 mM apigenin, even at 150 min after addition of the sperm extracts. We also monitored cumulus cell dispersal using capacitated epididymal sperm at a concentration of 150 cells/μl. Dispersal was strongly inhibited by apigenin, and most of the oocytes still retained cumulus cells abundantly 4 h after insemination. These results indicate that the inhibition of hyaluronan-degrading activity by apigenin results in retention of cumulus cells within the OCC.

To assess whether the hyaluronan-degrading activity is required for sperm penetration through the cumulus matrix, we carried out cumulus penetration assays in the presence of apigenin (Fig. 3A). Mouse epididymal sperm were labeled with Hoechst 33342, capacitated and mixed with the OCCs. After 15 min of incubation, the numbers of Hoechst-labeled sperm in the cumulus matrix and on the surface of oocyte ZP were counted (Fig. 3B). Capacitated epididymal sperm entered the OCC, traversed the cumulus matrix and reached the ZP surface in the absence of apigenin, as described previously [11]. The numbers of sperm in the cumulus matrix and on the ZP were found to be approximately 17 and 10 cells, respectively. Unexpectedly, in the presence of apigenin (0.5 and 1 mM), sperm were capable of reaching the ZP surface. The sperm numbers in the cumulus matrix and on the ZP were not affected significantly by the presence of apigenin. Similar results were obtained when Hoechst-labeled, capacitated sperm were incubated with the OCCs for 30 min, except that more sperm entered the cumulus matrix and reached the ZP (data not shown). Thus, the hyaluronan-degrading activity of sperm hyaluronidase may not be required for sperm penetration through the cumulus matrix. It should be noted that although we examined the OCCs 60 min after insemination, the data obtained was excluded owing to the fact that
MOUSE SPERM HYALURONIDASE

143

A large number of cumulus cells had already broken away from the oocytes in the absence of apigenin (also see Fig. 2).

Since epididymal sperm have the ability to penetrate through the cumulus matrix in the presence of apigenin (Fig. 3), it is intriguing to examine whether apigenin influences subsequent fertilization events, including sperm penetration through the ZP and sperm fusion with the oocyte. Capacitated epididymal sperm were mixed with the OCCs in the absence and presence of apigenin (0.5 or 1 mM), and the mixture was incubated for 1 and 4 h after insemination (Fig. 4). At the 4-h stage, cumulus cells were lost in most of the oocytes in the absence of apigenin. Apigenin resulted in the oocytes surrounded by widely varying numbers of cumulus cells; approximately 10–15% of the oocytes completely lost cumulus cells, whereas the other oocytes were surrounded by a large or small number of cumulus cells; and the mixtures were incubated for 1 or 4 h after insemination (Fig. 4). These results suggest that the sperm function may not be influenced by the apigenin-dependent inhibition of the hyaluronan-degrading activity of sperm hyaluronidase.

Discussion

This study demonstrates that the hyaluronan-degrading activity of sperm hyaluronidase is not required for the fertilization pro-
cesses, at least in vitro, including sperm entry into the OCC and passage through the cumulus matrix. To our knowledge, there have been no previous reports conclusively showing that a sperm protein(s) is directly responsible for sperm penetration through the cumulus matrix. It is thus reasonable to consider at present that sperm motility is an essential factor for cumulus penetration. We also speculate that sperm hyaluronidase may assist sperm in accessing the oocyte ZP not by the hyaluronan-degrading activity but by another function(s).

Partially inconsistent with the findings in monkey sperm [13, 14], mouse sperm are capable of normally penetrating the cumulus matrix in the presence of apigenin (Figs. 3 and 4). Even in monkey sperm, apigenin incompletely inhibits sperm penetration through the cumulus; the sperm ability to traverse the cumulus is reduced but is not abrogated by apigenin [13, 14]. In some experiments, we have examined the effects of apigenin on the motility and spontaneous acrosome reaction of sperm (data not shown). Apigenin did not affect these two cellular events of mouse sperm, as was the case for monkey sperm [13, 14]. These results raise the following question: besides sperm motility, what is required for sperm penetration through the cumulus matrix? Previously, we found that p-aminobenzamidine, a competitive inhibitor of serine proteases, inhibits sperm entry into the OCC and sperm penetration of the cumulus matrix [11]; only a very small number of sperm entered the OCC, and no sperm reached the ZP surface. Since the hyaluronan-degrading activity of sperm extracts is not inhibited by p-aminobenzamidine (data not shown), it is possible that p-aminobenzamidine-sensitive sperm proteases, including ACR [18] and PRSS21 [17], play a role in sperm entry into and penetration through the cumulus.

Our previous works using wild-type, Hyal5/−/− and Spam1/−/− epididymal sperm suggest that only SPAM1 may be implicated in sperm access to the oocyte ZP [11]. The function of Hyal5/−/− sperm is comparable to that of wild-type sperm. It is noteworthy that the loss of SPAM1 results in a remarkably increased accumulation of sperm on the surface or outer edge of the OCC, suggesting a possible involvement of SPAM1 in an initial step of sperm entry into the OCC [11]. In the present study, despite a strong inhibitory effect of apigenin on the hyaluronan-degrading activity of HYAL5 and SPAM1 (Figs. 1 and 2), wild-type epididymal sperm did not accumulate on the OCC surface (Fig. 3). These data imply that accumulation of Spam1/−/− sperm may result from a nonenzymatic function(s) of SPAM1, such as recognition of and binding to the OCC surface. Indeed, guinea pig SPAM1 has been reported to be bifunctional and to exhibit two distinct activities, the hyaluronan-degrading and ZP-binding activities exerted by the N- and C-terminal domains of the SPAM1 molecule [12]. However, in our examinations, mouse SPAM1 has failed to bind the solubilized ZP [9, 10]. At any rate, further experiments focusing on the components of the OCC surface are necessary to elucidate the unknown function of the C-terminal domain in SPAM1.

Our data emphasize that sperm hyaluronidase is not essential for fertilization, at least in the mouse. Even in Spam1/−/− mice, epididymal sperm still possess the ability to reach the oocyte ZP in vitro, despite the delays in cumulus cell dispersal and cumulus penetration. Thus, analysis of SPAM1/HYAL5 double-knockout mice will provide a definitive answer to the physiological function of sperm hyaluronidase in fertilization. Generation of SPAM1/ACR or SPAM1/PRSS21 double-knockout mice may also contribute to understanding the mechanism underlying sperm entry into the cumulus because p-aminobenzamidine-sensitive serine proteases of sperm presumably play a critical role in the sperm entry process in cooperation with SPAM1.

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