Factors Regulating Changes of Head-to-Head Agglutinability in Boar Spermatozoa During Epididymal Transit and Capacitation *In Vitro* - Review -

Hiroshi Harayama* and Seishiro Kato[1]
Department of Life Science, Graduate School of Science and Technology, Kobe University
Kobe 657-8501, Japan

ABSTRACT : In boar spermatozoa, the head-to-head agglutinability changes in parallel with the development of the fertilizing ability. Namely, both abilities gradually increase in the distal caput and corpus epididymides, but are subsequently suppressed in the cauda epididymidis. It has been postulated that these changes of the agglutinability are controlled via sperm interaction with specific epididymal plasma factors including agglutination mediators (agglutinins) and inhibitors (anti-agglutinins). Expression of these abilities (sperm agglutination and capacitation) is hardly observed in spermatozoa immediately after ejaculation, but it occurs during incubation in a capacitation medium. Recently, we have purified and characterized epididymal plasma anti-agglutinin for boar spermatozoa. Moreover, we have conducted a series of experiments to reveal biological significance and mechanism of the head-to-head agglutination and have accumulated data indicating that boar sperm agglutination is mediated by capacitation-supporting factors including calcium, bicarbonate and sterol acceptors. This review introduces our recent data and discusses a possible mechanism for suppression of the agglutinability in the distal epididymidis and relationship between agglutinability and fertilizing ability. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 8 : 1196-1202)*

Key Words : Boar, Cell Agglutination, Epididymal Protein, Sperm Maturation, Capacitation

INTRODUCTION

It has been established that the epididymis produces a favorable environment for the maturation and storage of luminal spermatozoa by epithelial secretion and absorption (Bedford, 1975; Orgebin-Crist et al., 1975; Hammerstedt and Parks, 1987). In boars, spermatozoa gradually acquire the capacities to move progressively (Dacheux et al., 1983; Harayama and Kato, 1992), to undergo capacitation (Hunter and Polge, 1986), and to interact with zona pellucida (Peterson et al., 1986) and egg plasma membrane (Dacheux et al., 1983; Harayama et al., 1993) during epididymal transit. Consequently most cells develop fertilizing ability in the distal caput and corpus epididymides (Holtz and Smidt, 1976; Yanagimachi, 1994). In the cauda epididymidis, however, sperm fertilizing ability appears temporarily suppressed by actions of the stabilizing factors for the storage. These factors are contained in cauda epididymal plasma (Hunter et al., 1976, 1978), but they have not been characterized yet. Likewise, boar spermatozoa exhibit changes in the head-to-head agglutinability during epididymal transit; this ability gradually increases in the distal caput and corpus epididymides and is subsequently suppressed in the cauda epididymidis (Dacheux et al., 1983). Thus, we regard these changes of the head-to-head agglutinability as one aspect of sperm maturation. The changes appear controlled via the addition of sperm agglutination mediators (agglutinins) and inhibitors (anti-agglutinins) from the epididymis to the luminal spermatozoa and epididymal plasma; several epididymal proteins have been identified as agglutinins (Flaherty et al., 1993; Fornes and Burgos, 1994) and as anti-agglutinins (Roy and Majumder, 1989) in some animal species. However, only a few attempts have been made at identifying these factors in boars (Dacheux et al., 1983).

After ejaculation, spermatozoa gradually become capable of fertilizing oocytes during the stay in the specific environment that is provided by the female reproductive tract. This event is called "capacitation". Various components on the surface and in the intracellular space of spermatozoa are altered during capacitation in order to activate molecules required for expression of the fertilizing ability. The environment within the female reproductive tract can be simulated by incubation in an appropriate medium (a capacitation medium) *in vitro* (Yanagimachi, 1994). It is also observed that many spermatozoa become agglutinated with one another at the acrosome during the incubation (Harayama et al., 1998). So, we have a hypothesis that sperm agglutination is one of the events associated with capacitation.

In this review, we introduce our experimental results concerning a boar epididymal plasma protein "anti-agglutinin" and effects of capacitation-supporting factors on boar sperm head-to-head agglutination. Based on our data, we also discuss a possible mechanism for suppression of the agglutinability in the distal epididymidis and relationship

* Address reprint request to Hiroshi Harayama, Tel: +81-78-803-5805, Fax: +81-78-803-5807, E-mail: harayama@ans.kobe-u.ac.jp.
[1] Department of Animal Science, Faculty of Agriculture, Kobe University, Kobe 657-8501, Japan.

between agglutinability and fertilizing ability in boar spermatozoa.

SUPPRESSION OF SPERM HEAD-TO-HEAD AGGLUTINABILITY IN THE DISTAL EPIDIDYMIDIS

Identification and biochemical characterization of epididymal protein anti-agglutinin

In our first experiment (Harayama et al., 1994), we assessed the head-to-head agglutinability of boar spermatozoa collected from the different regions of the epididymis. When boar spermatozoa from the corpus epididymidis were diluted and incubated in a capacitation medium for 1 h, most were agglutinated with one another at the acrosome in groups of more than ten cells. By contrast, the same treatment of dilution and incubation hardly induced agglutination in cauda epididymal spermatozoa. These observations confirm that the agglutinability of boar spermatozoa is suppressed in the distal epididymidis, as described previously (Dacheux et al., 1983). However, the washing treatment and prolonged incubation greatly promoted the head-to-head agglutination in cauda epididymal spermatozoa. These results indicate that an anti-agglutinin(s) is present in boar cauda epididymal fluid and is at least partially removed from the epididymal spermatozoa by the washing and subsequent incubation.

Cauda epididymal plasma contains a variety of factors inhibiting sperm function, such as a sperm-motility quiescence factor in bulls (Carr and Acott, 1984), immobilin in rats (Usselman and Cone, 1983) and acrosome stabilizing factor in rabbits (Eng and Oliphant, 1978; Reynolds et al., 1989). The environment within the epididymal duct is rendered unfavorable for the sperm movement and premature spontaneous acrosome reaction by these molecules. We (Harayama et al., 1994) observed that the intact cauda epididymal plasma effectively inhibited the occurrence of head-to-head agglutination in boar spermatozoa. Moreover, the anti-agglutination activity was maintained in the fraction precipitated from the epididymal plasma with cold ethanol, but was abolished in epididymal plasma after heat treatment. These findings indicate that cauda epididymal plasma contains a protein(s) with the anti-agglutination activity for spermatozoa (anti-agglutinin(s)). Dacheux and Dacheux (1987) have purified a 250-kDa protein with the anti-agglutination activity (a high-molecular-mass anti-agglutinin: a HMM anti-agglutinin) from boar epididymal plasma. This protein is composed of four 63-kDa subunits and is secreted from the principal cells of the caput and corpus epididymides. Moreover, it has been identified as β-*N*-acetyl-hexosaminidase by analysis of the N-terminal amino acid sequence (Syntin et al., 1996). Recently, we (Harayama et al., 1994) also purified another anti-agglutinin from boar cauda epididymal plasma that was a protein with a SDS-PAGE mobility of 25 kDa (a low-molecular-mass anti-agglutinin: a LMM anti-agglutinin).

Our data on biochemical characterization of the LMM anti-agglutinin (Harayama et al., 1994; 1996; 2000) are summarized in table 1. The molecular mass estimated by SDS-PAGE under both the reduced and non-reduced conditions was 25 kDa that was in agreement with the molecular size estimated by gel filtration (25-29 kDa). However, the molecular masses measured by matrix-

Table 1. Biochemical characterization of boar epididymal LMM anti-agglutinin[a]

		References
Molecular mass		
SDS-PAGE (Non-reduced)	25 kDa	unpublished data
SDS-PAGE (Reduced)	25 kDa	Harayama et al., 1994
Gel filtration	25-29 kDa	Harayama et al., 1994
MALDI-TOF/MS[b] (Intact)	Approximately 18,900-19,600 Da	unpublished data
ESI-MS[c] (Intact)	Two major peaks (19,379 Da, 19,395 Da) and several minor peaks (e.g., 19,088 Da, 19,104 Da, 19,297 Da)	Harayama et al., 2000
ESI-MS[c] (Deglycosylated and tryptic digested)	Four fragments (12,668 Da, 5,209 Da, 1,226 Da, 1,168 Da)	Harayama et al., 2000
Isoelectric points	One major form (5.8) and two minor forms (5.6, 5.95)	Harayama et al., 1996
Posttranslational modification	Sialylation in the major form and phosphorylation at the serine and/or threonine residues	Harayama et al., 1996, 2000
Molecule binding to sperm	Major form	Harayama et al., 1996
N-terminal amino acid sequence	KTDDY AISGA KEEEF YDYME ELYAV	Harayama et al., 2000

[a] Examined by SDS-PAGE, two-dimensional PAGE, blotting techniques (Western, lectine, ECL glycoprotein detection kit), gel filtration, mass spectrometry and N-terminal amino acid sequence anaysis.

[b] Matrix-assisted laser desorption-time of flight/mass spectrometry.

[c] Electrospray ionization-mass spectrometry.

assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS: 18,900-19,600 Da) and electrospray ionization-mass spectrometry (ESI-MS: major peaks 19,379 Da and 19,395 Da) were different from those estimated by SDS-PAGE. It is likely that posttranslational modifications (Jones et al., 1989) and different amino acid compositions (de Jong et al., 1978; Noel et al., 1979) limit the estimation of the exact molecular mass by SDS-PAGE. We also observed that the LMM anti-agglutinin was phosphorylated at serine and/or threonine residues and that dephosphorylation of this protein with alkaline phosphatases shifted a SDS-PAGE mobility from 25 kDa to approximately 19.5 kDa. Thus, structural modifications of the LMM anti-agglutinin by phosphorylation might cause overestimation of molecular mass by SDS-PAGE.

Mass spectra of the LMM anti-agglutinin obtained by ESI-MS were characterized by two major and several minor peaks. The LMM anti-agglutinin, apparently homogenous in the SDS-PAGE pattern, was separated into one major (pI 5.8, 25 kDa) and two minor forms (pI 5.6, 25 kDa and pI 5.95, 25 kDa) by the two-dimensional PAGE. Moreover, sialic acid residues were present in the major form (pI 5.8, 25 kDa), but not in the minor forms (pI 5.6, 25 kDa and pI 5.95, 25 kDa). These findings indicate molecular heterogeneity in the LMM anti-agglutinin. Similar molecular heterogeneity of the male reproductive tract fluid proteins has been reported in bovine seminal plasma proteins (Desnoyers et al., 1994) and a 135-kDa boar epididymal plasma protein (Okamura et al., 1992). These examples of molecular heterogeneity appear due to the modifications after translation (Desnoyers et al., 1994) and/or alternative splicing of the mRNA (Freemerman et al., 1995). Our data suggest that posttranslational differences in sialylation may account for the micro heterogeneity between the major and minor forms of the LMM anti-agglutinin, found by the two-dimensional PAGE. The major form (i.e., sialylated form) of the LMM anti-agglutinin (pI 5.8, 25 kDa) was associated with epididymal spermatozoa. Epididymal sialoproteins have been detected in a variety of animal species and have been associated with maturation-dependent modifications of the sperm surface in the epididymis (Eddy and O'Brien, 1994). Especially, the sperm maturation sialoprotein (called antigen 4 or T21 antigen) is secreted from the distal caput and proximal corpus epididymides of mice and is bound onto the spermatozoa (Eddy and O'Btien, 1994). This antigen inhibits the occurrence of tail-to-tail agglutination in mouse epididymal spermatozoa (Feuchter et al., 1988), and its sialic acid residues appear related to this function, as desialylation by neuraminidase treatment causes rapid sperm agglutination (Toshimori et al., 1990). It has been generally accepted that sialoproteins on the surface of various cells are involved directly and indirectly in the cell-to-cell interaction including agglutination and disagglutination (Jeanloz and Codington, 1976; Schauer, 1985). These imply possible involvement of sialic acid residues in the activity of the LMM anti-agglutinin.

Additionally, ESI-MS of fragments trypsin-digested after deglycosylation and the amino acid sequence analysis revealed that the LMM anti-agglutinin had a unique peptide-mass fingerprinting of fragments and a novel N-terminal amino acid sequence, respectively. These findings suggest that the LMM anti-agglutinin may be a novel protein.

Immunolocalization of LMM anti-agglutinin

Immunocytochemical observation of boar spermatozoa revealed that the LMM anti-agglutinin was associated uniformly with the acrosome and equatorial segment, but not with the postacrosomal region. In some spermatozoa, this protein was also located partially on the flagellum (Harayama et al., 1999a). When boar epididymal spermatozoa were washed and incubated, many of the cells were agglutinated with one another at the acrosome, as described above. Probably, the association of the LMM anti-agglutinin with the acrosome is important in the inhibition of the sperm head-to-head agglutination.

Our data on the localization of the LMM anti-agglutinin in boar epididymis (Harayama et al., 1995, 1999a) are summarized in table 2. The LMM anti-agglutinin was first detected in epididymal plasma from the proximal corpus and more intensively in the more distal regions, indicating that free LMM anti-agglutinin appears in the proximal corpus, with levels increasing progressively in the distal epididymidis. The origin of the LMM anti-agglutinin appears to be the corpus epididymidis as this antigen was detected in the epithelia of this region by indirect immunofluorescence. On the other hand, in the samples extracted from the epididymal spermatozoa in the SDS-PAGE sample buffer containing 1% SDS and 2.5% 2-mercaptoethanol, the LMM anti-agglutinin was detected in the regions between the proximal corpus and distal cauda, with the relatively higher level found in the distal corpus. By contrast, different detection patterns of the LMM anti-agglutinin were obtained when sperm proteins were extracted in a SDS-PAGE sample buffer containing 2% SDS and 5% 2-mercaptoethanol. A stronger reaction of the LMM anti-agglutinin with the specific antiserum was detected in the extracts from the distal cauda epididymal spermatozoa than in those from the distal corpus epididymal spermatozoa. These observations suggest a firmer association of the LMM anti-agglutinin with the cauda epididymal spermatozoa than with the corpus epididymal spermatozoa.

Western blotting of the tissue extracts from twelve kinds of organs (testis, epididymis, seminal vesicle, prostate, heart, liver, kidney, spleen, stomach, small intestine, lung and

Table 2. Immunolocalization of boar epididymal LMM anti-agglutinin in the epididymis[a]

	Caput		Corpus		Cauda			References
	Proximal	Distal	Proximal	Distal	Proximal	Central	Distal	
Western blotting								
Epididymal plasma[b]	−	−	±	+	+		+ +	Harayama et al., 1995
Epididymal plasma[c]		−	+			+ +		Harayama et al., 1999a
Western blotting								
Sperm extracts[b]	−	−	±	+	±		±	Harayama et al., 1995
Sperm extracts[c]				+			+ +	Harayama et al., 1995
Indirect immunofluorescence								
Frozen sections of epididymides (Epithelia)	−	−	+	+		±		Harayama et al., 1999a
Frozen sections of epididymides (Luminal contents)	−	−	+	+		+		Harayama et al., 1999a

[a] Examined by Western blotting techniques after SDS-PAGE and indirect immunofluorescence.
[b] Treated in the SDS-PAGE sample buffer containing 1% SDS and 2.5% 2-mercaptoethanol.
[c] Treated in the SDS-PAGE sample buffer containing 2% SDS and 5% 2-mercaptoethanol.

muscle) revealed that the LMM anti-agglutinin was detected only in the corpus and cauda epididymides (Harayama et al., 1999a). This suggests that the LMM anti-agglutinin may be restricted to the male reproductive tract in boars. However, it has been reported that some homologues of proteins are generated as a result of varying modifications after the translation and alternative splicing of mRNA (Desnoyers et al., 1994; Freemerman et al., 1995). The presence of the homologue that is non-reactive with the specific antiserum has not yet been determined.

OCCURRENCE OF SPERM AGGLUTINATION DURING CAPACITATION *IN VITRO*

Fate of LMM anti-agglutinin after ejaculation

The free anti-agglutinin was detected in both epididymal and seminal plasma. However, its concentration greatly declined at ejaculation, due to the addition of secretive fluids from the accessory genital glands (Harayama et al., 1996, 2000). The sperm-bound LMM anti-agglutinin was abundantly detected in the cells immediately after ejaculation, but gradually released from the cells during the capacitation *in vitro* (Harayama et al., 1999b).

Effects of capacitation-supporting factors on sperm agglutination

A number of capacitation media have already been reported for spermatozoa from several animal species. Many of them contain the common compounds; especially, calcium, bicarbonate and serum albumin have been considered to act as capacitation-supporting factors (Yanagimachi, 1994). In this section, we have described effects of these factors on sperm head-to-head agglutination and proposed its possible mechanism.

Evidence obtained in the previous reports (reviewed by Fraser and Monts, 1990; Visconti and Kopf, 1998) strongly indicates that calcium rises an intracellular concentration of cyclic adenosine 3',5'-monophosphate (cAMP) leading to capacitation in rodent spermatozoa by stimulation of the adenylyl cyclases. Moreover, there is also evidence for the presence of a calcium/calmodulin-dependent phosphodiesterase that is modulated indirectly by actions of calcium in rodent spermatozoa. However, actions of calcium to these enzymes are understood less clearly in capacitating boar spermatozoa. We (Harayama et al., 1998) observed that the head-to-head agglutination in boar spermatozoa was dependent on the calcium concentrations of the capacitation medium and occurred most intensively at the calcium concentrations similar to those of seminal plasma (Setchell et al., 1994) and female reproductive tract fluids (Iritani et al., 1974). Moreover, the agglutinated spermatozoa were dispersed by the chelation of the extracellular calcium. Harrison et al. (1993) have observed a considerable influx of the calcium in boar spermatozoa incubated in a capacitation medium by flow cytometry. This calcium influx appears to be a result of specific destabilization of the plasma membrane by incubation with serum albumin and bicarbonate. Ruthenium red inhibits the calcium influx in boar spermatozoa by possible actions to the plasma membrane (Peterson et al., 1983). In our experiments, this reagent significantly reduced occurrence of the head-to-head agglutination in boar spermatozoa (Harayama et al., 1998). Moreover, the addition of nifedipine (1 mM) or nitrendipine (1 mM) to a capacitation medium attenuated the effects of calcium on head-to-head agglutination

(unpublished data). Nifedipine and nitrendipine have been reported to act as blockers of a dihydropyridine-type voltage-sensitive calcium channel (Aosaki and Kasai, 1989; Skeen et al., 1993). Considering these findings along with the above-mentioned calcium dependence, we believe that sperm head-to-head agglutination may be stimulated by the calcium influx through the calcium channel in the plasma membrane.

Bicarbonate passes into the intracellular space of spermatozoa through anion transporters in the plasma membrane (Okamura et al., 1988) including bicarbonate/chloride exchangers (Parkkila et al., 1993; Holappa et al., 1999) and sodium/bicarbonate cotransporters (Jensen et al., 1999), and then regulates intracellular pH. It also stimulates the activity of the sperm adenylyl cyclases, resulting in increased generation of the signaling second messenger (Okamura et al., 1985). The adenylyl cyclases in the spermatozoa synthesize cAMP from ATP, as described in somatic cells. However, a soluble form of them possesses a unique molecular structure and is apparently distinct from a classical transmembrane form usually found in other cells (Buck et al., 1999; Sinclair et al., 2000; Chen et al., 2000; Kaupp and Weyand, 2000). This soluble form is originally synthesized as a precursor in the testis followed by proteolytical cleavage into several fragments including the soluble form during sperm transit through the epididymis. Control of the enzyme activity is also unique in the spermatozoa; the soluble form does not require G-protein for the activation, but is stimulated by the direct interaction with bicarbonate. It is likely that the increased cAMP in spermatozoa induces protein tyrosine phosphorylation probably associated with the capacitation through the modulation of the enzymes including cAMP-dependent protein kinase, protein tyrosine kinase and protein tyrosine phosphatase (Visconti and Kopf, 1998). We observed that replacement of sodium bicarbonate with HEPES in a capacitation medium significantly reduced occurrence of head-to-head agglutination in boar spermatozoa after incubation (Harayama et al., 1998). This suggests that bicarbonate may be a pivotal factor regulating the occurrence of the sperm head-to-head agglutination. Possible bicarbonate-mediated signaling cascade leading to the sperm agglutination still remains to be investigated.

Effects of serum albumin on capacitation have already been observed in spermatozoa from various animal species, and mechanisms for promotion of capacitation by this protein have been elucidated. The release of cholesterol from the sperm plasma membrane is mediated by the membrane's interaction with serum albumin, leading to changes in membrane architecture and fluidity which give rise to the capacitation state (Cross, 1998; Visconti et al., 1999a,b). This event is mimicked by the treatment with β-cyclodextrin (Visconti et al., 1999a,b), which is a cyclic heptasaccharide consisting of β(1-4)-glucopyranose units (Pitha et al., 1988). Loss of the anti-agglutinin from the acrosome is important for the head-to-head agglutination in boar spermatozoa (Harayama et al., 1996, 1999a,b). Our recent report (Harayama et al., 2001) revealed that sterol acceptors including serum albumin and β-cyclodextrin enhanced boar sperm agglutination and promoted the loss of sperm-bound anti-agglutinin. However, premixing these reagents with a cholesterol analogue to saturate their cholesterol-binding sites reduced their effects on sperm agglutination and on the loss of sperm-bound anti-agglutinin. These findings indicate the effects of the sterol acceptors are dependent at least partially on their ability to capture cholesterol from the sperm plasma membrane. Thus, the loss of sperm-bound anti-agglutinin might be modulated by changes in the plasma membrane induced by the membrane's interaction with the sterol acceptors contained in a capacitation medium. In addition, a variety of lipid-binding proteins have been isolated from the fluids of the male and female reproductive tracts, including BSP proteins (Thérien et al., 1999), high-density lipoprotein (Ehrenwald et al., 1990) and HE1 (Okamura et al., 1999). Biochemical characterization indicates that these molecules could capture cholesterol from the sperm plasma membrane. Possibly, these proteins are also regulatory factors supporting sperm agglutination in the reproductive tract.

Possible relationship between sperm agglutinability and fertilizing ability

A precise relationship between sperm agglutinability and fertilizing ability has not been established yet. However, we have been accumulating evidence suggesting a possible link between these abilities. For example, 1) changes of the head-to-head agglutinability occur in parallel with the development of the fertilizing ability in the epididymis (see introduction). 2) Sperm agglutination is stimulated by capacitation-supporting factors including calcium, bicarbonate and sterol acceptors (Harayama et al., 1998, 2001). 3) The addition of seminal plasma containing decapacitation factors to a capacitation medium attenuates head-to-head agglutination, which is caused by decreased loss of the anti-agglutinin from the sperm surface (Harayama et al., 1999b). 4) When boar spermatozoa are incubated in a capacitation medium, the percentages of head-to-head agglutinated cells show a significant correlation with those of capacitated cells (Harayama et al., 2001). These findings could be interpreted as showing that sperm head-to-head agglutination is related to changes in boar spermatozoa during capacitation.

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