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Loss of Surface-Associated Albumin during Capacitation and Acrosome Reaction of Mouse Epididymal Sperm *in vitro*

Myung Chan Gye and Moon Kyoo Kim*

Department of Biology, College of Natural Sciences, Hanyang University, Seoul 133-791, Korea

In order to examine the interaction of albumin with the sperm during capacitation in mouse, proteins of cauda epididymal sperm were extracted under various conditions and analyzed with SDS-PAGE. Sperm surface labeling patterms were also examined using fluorochrome-conjugated wheat germ agglutinin (WGA) and bovine serum albumin (BSA). Albumin was detached from the sperm surface during the incubation and seemed to be constituted the major protein components of the conditioned media in which sperm incubated for 90 min. Detachment of albumin from the sperm was not affected by the Ca^{2+} in the medium. WGA-FITC labeling confirmed that Triton X-100 permeabilized plasma membrane overlaying the apical segment of sperm head and detached plasma membrane associated proteins having negatively charged glycoconjugates. BSA-FITC labeling of epididymal sperm occurred on the apical segment of periacrosomal region and postacrosomal region of the head. BSA-FITC labeling was not observed in periacrosomal region of the sperm treated with Ca^{2+}-ionophore A23187 (10 μM), whereas the postacrosome region of acrosome-reacted sperm was still labeled after the AR. These results suggest that albumin bound to the surface of epididymal sperm is detached during the capacitation process, and it might be involved in physiological change of sperm plasma membrane accompanying the capacitation.

KEY WORDS: Albumin, Capacitation, Acrosome Reaction, Sperm, Mouse

Testicular sperm undergoes "maturation" during the epididymal transit. After ejaculation, sperm still needs a final maturation process in the female genital tract, collectively described as sperm capacitation, to obtain the ability to fertilize the egg (Austin, 1951; Chang, 1951). Capacitated sperm binds to zona pellucida (ZP) and undergoes acrosome reaction (AR) in a Ca^{2+}-dependent manner, penetrates the investments of oocyte, and eventually fuse with oolemma (Yanagimachi, 1994).

The plasma membrane of the sperm head is largely divided into two regions, periacrosomal and postacrosomal domains. Periacrosomal domain consists of three subregions corresponding to the apical, principal, and equatorial segment of the acrosome (Fig. 1). The plasma membrane over the apical and principal segment contains receptor for ZP and participates in the membrane fusion events of the acrosome reaction, whereas the plasma membrane over the equatorial segment may function in the fusion with egg plasma membrane (Talbot, 1985). During the sperm maturation process, extensive changes take place in plasma membrane of spermatozoa. Protein redistribution occurrs in the plasma membrane of

*To whom correspondence should be addressed.

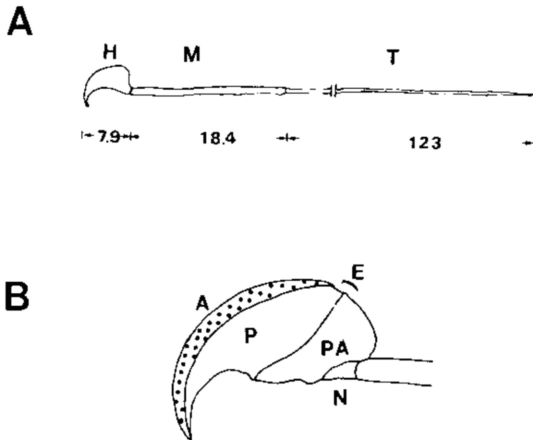

Fig. 1. Schematic drawing of the mouse mature spermatozoon. (A) Entire sperm showing dimensions in micrometer. (B) Domains in sperm head. Abbvrations: H, head; M, middle piece; T, tail; A, apical segment; P, principal segment; E, equatorial segment; PA, postacrosomal region; N, neck. Adopted from Olson and Winfrey (1991).

sperm head during the post-testicular maturation (Suzuki and Yanagimachi, 1986). Changes in protein mobility (Koehler, 1976; Flechon, 1985) and lipid composition (Langlais and Robert, 1985) are evident during the capacitation. Fluid in the male reproductive tract such as seminal vesicle fluid contain acrosome reaction inhibiting factors, and these factors are associated with sperm surface during the sperm transit through the tract. Sperm capacitation *in vitro* involves loss of surface-associated components (Hartmann and Gwatkin, 1971; Fraser, 1984) such as decapacitation factors (Fraser *et al.*, 1990), acrosome stabilizing factor (Davis and Davis, 1983), and coating materials from male accessory sex glands (Oliphant, 1976; Oliphant and Singhas, 1979). Capacitation is regarded as a prerequisite for acrosome reaction, a membrane fusion event, therefore studies on the biochemical properties of sperm surface during the differentiation of spermatozoa are important for the elucidation of acrosome reaction mechanism as well as sperm-egg interaction during early step in fertilization.

The amount of time required for the capacitation depends on the concentration of albumin (Ward and Storey, 1984). Bovine serum albumin (BSA) was widely used as a medium supplement for the capacitation of sperm. BSA itself posesses lipid transfer activity and with the activity BSA promotes the sperm capacitation (Stewart-Savage, 1993). Previous studies on the interactions of albumin with sperm surface were supported only by rather indirect evidences. By using the cytological assessment such as chlorotetracyclin- (Ward and Storey, 1984) and Coomassie dye (Moller *et al.*, 1990) staining, AR inducibility was assessed after incubation of sperm in various concentrations of albumin (Ward and Storey, 1984). However, it has not yet been elucidated whether the binding property of sperm plasma membrane to albumin is altered during sperm capacitation and AR. Milimolar concentration of Ca^{2+} is required for AR but not for capacitation (Fraser, 1987). But it has not been determined whether the interaction between the albumin and sperm requires extracellular Ca^{2+}.

To verify the capacitation promoting effect of albumin, evidences that albumin binds to and subsequently detaches from the sperm surface during the capacitation are needed. Therefore we analyzed sperm proteins by differential extraction before and after incubation with or without Ca^{2+}. Labeling patterns of fluorochrome-conjugated WGA and BSA to sperm head surface were monitored.

Materials and Methods

Sperm preparation: For labeling experiments, testis and epididymis were removed from 3 month-old male mouse (ICR strain) and the blood was cleared from tissues by blotting to filter paper. Testicular sperm was collected by triple filtration through the lens paper. Caudal portion of epididymis was further dissected according to Origebin-Crist *et al.* (1981). Dissected tissues were squeezed to expell its contents with forcep in the modified Tyrode solution (Parrish *et al.*, 1988) without Ca^{2+}. Epididymal sperm suspension was collected after 10 min. Sperm concentration was adjusted to 2×10^6 sperm/ml with fresh medium and 1 ml of aliqouts were preincubated in 5% CO_2

and 95% air at 37°C for 90 min. Ca^{2+} stock solution (171 mM $CaCl_2$) was added to preincubated sperm suspension with final concentration of 1.71 mM. After 10 min, Ca^{2+}-ionophore A23187 (10 μM in 0.1% DMSO) was added incubated for further 30 min to induce AR.

Protein analysis: Epididymal sperm suspension was washed with 10 times vol. of PBS by centrifugation at 200 g for 10 min 3 times. The sperm pellet was resuspended in 1 ml of extraction buffer (0.1% Triton X-100 in PBS), incubated for 2 hr at 4°C, and centrifuged at 20,000 g for 30 min. After centrifugation, the supernatant was saved and the remaining sperm pellet was resuspended in 1 ml PBS and then lysed with the same volume of 2X Laemmli sampling buffer. The culture supernatant was collected from sperm suspension preincubated for 90 min by centrifugation of sperm suspension at 20,000 g for 30 min at 4°C. Epididymal fluid was prepared from the supernatant after the first wash of epididymal spermsuspension. Cell debris was further cleared through the millipore filtration, Filter-through was concentrated into 100 ul using the Centricon (Amicon, M.W. cut 10,000 Da). Fresh epididymal sperm, capacitated sperm and acrosome-reacted sperm were collected after proper incubation. They were centrifuged at 20,000 g for 30 min and supernatants were discarded. 100 μl of Tris buffered saline containing 0.1% (v/v) Triton X-100 was added to sperm pellets, resuspended, and incubated for 20 min at 4C. Concentrated protein solutions were mixed with same vol. of 2X Laemmli (1970) sample buffer and boiled for 10 min. SDS-PAGE was performed in 5-20% gradient gel or 10% gel. After PAGE, gels were silver stained according to the method by Merril *et al.* (1981).

Lectin labeling: wheat germ agglutinin (WGA) solution (1 mg/ml) was purchased from Vector laboratory. Lectin solution was diluted with in PBS (100 μg/ml). Equal volume of sperm suspension and lectin solution were mixed and incubated for 20 min at RT. Next procedures were done as described above. Labeling specificity of WGA-FITC was verified by incubating the sperm in the presence of 0.1 M *N*-acetylglucosamine (GlcNAc) as a hapten sugar (Kobata and Yamashita, 1993).

BSA-FITC labeling: Lyophylized BSA-FITC purchased from Sigma was dissolved in PBS (100 μg/ml). Equal volume of sperm suspension and BSA-FITC solution were mixed and incubated for 20 min at RT. The labeled sperm was washed with PBS twice. Wet mounts were made and sealed with nail vanish and observed under epi-fluorescence microscope (Zweiss Axioscope) using FITC filter block. Labeling specificity of BSA-FITC was verified by incubating the sperm in the medium containing excess amount of unlabeled BSA (5 mg/ml).

Results

Protein gel profile: Albumin was the major band commonly present in the Triton X-100-extracted fraction, epididymal fluid, and conditioned medium in which the sperm was incubated for 90 min (Fig. 2A). The bands present in the lane derived from Triton X-100-extracted fraction were also found in lanes of the conditioned medium and epididymal fluid. Two protein bands with M.W. of 20,000 - 26,000 were enriched in double-extracted fraction (SW*). These proteins were almostly absent in Triton X-100 extracted fraction (ST). As shown in the Fig. 2 B, uncapacitated sperm was rich in associated albumin but reduced amount of albumin was observed in the capacitated sperm and acrosome-reacted sperm. Albumin detachment was not affected by the Ca^{2+} in the medium (data not presented).

WGA binding: sWGA-FITC strongly bound to the apical segment of cauda epididymal sperm (Fig. 3A). However, principal segment was free from labeling. Middle piece and tail region were also labeled with same intensity. The pattern of labeling on the head of epididymal sperm pretreated 0.1% Triton X-100 for 20 min was occurred in the principal segment (Fig. 3C), and it was not different from that of acrosome-reacted sperm (Fig. 3B). At higher magnification, plasma membrane overlaying the apical segment showed faint fluorescence (Fig. 3C′). Presence of excess hapten sugar during labeling procedure decreased the labeling intensity, and only the faint

fluorescence was observed in the postacrosomal region of the sperm head surface (data not shown).

BSA binding: BSA-FITC weakly bound to the apical segment of testicular sperm with small extent (Fig. 4A). However, cauda epididymal sperm was extensively labeled in sperm surface covering the apical segment and postacrosomal region, middle piece and tail region (Fig. 4B). The pattern of labeling on the head of epididymal sperm preincubated for 90 min was not different from that of uncapacitated sperm (Fig. 3C). In the acrosome-reacted sperm, periacrosome region was not labeled, whereas postacrosome region was still labeled (Fig. 4D). Presence of excess unlabeled BSA during labeling procedure limited the labeling to postacrosome region, and no difference between acrosome-intact and acrosome-reacted sperm was observed (data not shown).

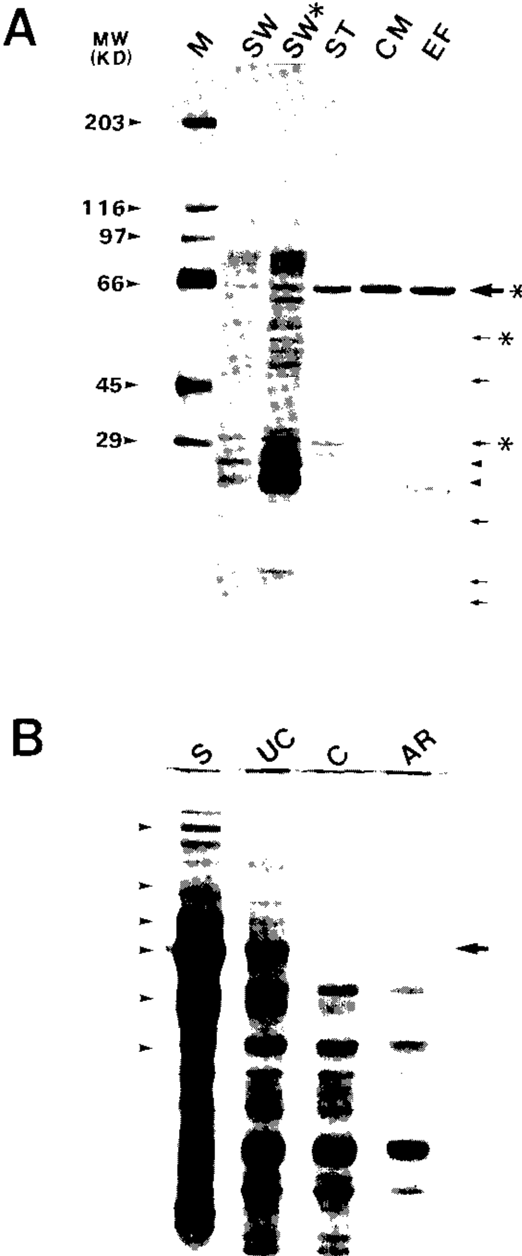

Fig. 2. SDS-PAGE analysis of epididymal sperm proteins. (A) Detergent (0.1% Triton X-100) solubilized sperm proteins of 2 × 10^6 cells or whole sperm lysate solubilized in Laemmli sample buffer were subjected to SDS-PAGE on 4-20% gradient gel and silver stained. M, marker proteins. SW, whole lysate of epididymal sperm. SW*, lysate of sperm pellet previousely extracted with 0.1% Triton X-100; ST, sperm proteins extracted with 0.1% Triton X-100; CM, conditioned media; EF, epididymal fluid. Large arrow indicates albumin fraction. Small arrows indicate proteins commonly present both in the epididymal fluid and Triton X-100 extracted fraction. Asterisks indicate protein bands commonly present among the epididymal fluid, conditioned media, and Triton X-100 extracted fraction. Arrowheads indicate proteins enriched in SDS fraction after Triton X-100 extraction. (B) Detergent (0.1% Triton X-100) solubilized sperm proteins previousely incubated under varying conditions were subjected to SDS-PAGE on 10% gel and silver stained. S, 1/100 diluent of serum. UC, uncapacitated epididymal sperm. C, sperm incubated for 90 min for capacitation. AR, acrosome-reacted sperm. Protein molecular weight markers are the same as (A). Arrow indicates albumin band.

Discussion

Results from this study showed that some of sperm associated proteins from epididymal origin are released from spermatozoa during the capacitation process in vitro. Many of protein bands in the Triton X-100 extracted fraction were found in both of the epididymal fluid and conditioned media in which the sperm was incubated for 90 min. It suggests that conditioned media are enriched with proteins detatched from sperm. Albumin fraction was the major protein

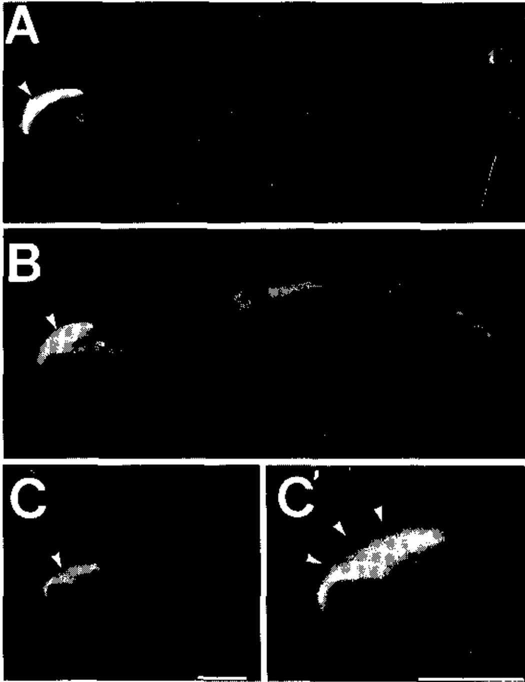

Fig. 3. Wheat germ agglutinin-FITC labeling patterns on sperm surface. Cauda epididymal sperm were incubated with WGA (50 μg/ml) for 20 min at RT. After labeling, sperm were washed twice with PBS, wet mounted and observed under epifluorescence microscope. (A) Cauda epididymal sperm. Intense labeling occured in the apical segment (arrowhead). (B) Acrosome-reacted sperm in the presence of A23187 (10 μM). Labeling pattern was similar to demembranated sperm in (B). Vesiculation of plasma membrane and outer acrosomal membrane is seen (arrowhead). (C) Caudal epididymal sperm treated with 0.1 % Triton X-100 for 20 min in PBS. Intense labeling was observed in principal segment of periacrosomal region (arrowhead). (C') Enlarged view of (C). Plasma membrane overlaying the apical segment showed faint fluorescence (arrowheads). Bar = 5 μm.

bands of proteins commonly found in the Triton X-100-extracted fraction (ST and U), in the conditioned media, and the epididymal fluid (Fig. 2). On the other hand, relatively small amount of albumin was found in the sperm previousely extracted with Triton X-100 for 2 hr (SW*). Having been not confirmed immunologically in this experiment, albumin was reported to be present major protein constituent in the epididymal fluid (Orgebin-Crist *et al.*, 1981). Decrease of extractable albumin of the capacitated

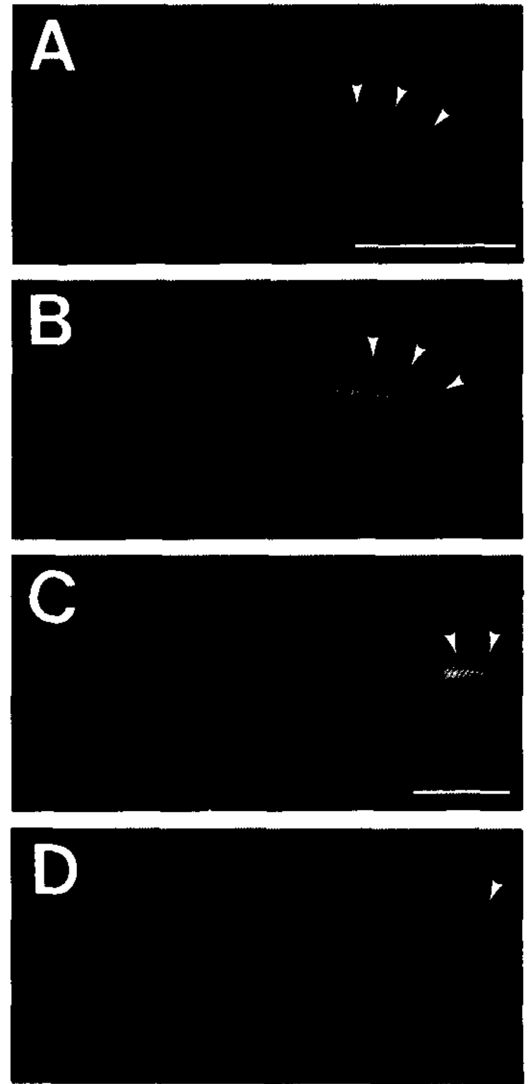

Fig. 4. BSA-FITC labeling patterns on sperm surface. Testicular, epididymal, and in vitro incubated sperm were incubated with BSA-FITC (50 μg/ml) for 20 min at RT. After labeling, sperm were washed twice, wet mounted and observed under epifluorescence microscope. (A) Testicular sperm, faint labeling occured in acrosome region. (B) Caudal epididymal sperm, intense labeling was observed in apical segment of periacrosomal region (arrowhead) and postacrosomal region, but not in the principal segment. (C) Cauda epididymal sperm incubated for 90 min. Labeling pattern was similar to uncapacitated sperm in (B). (D) Acrosome-reacted sperm. Labeling was not observed in periacrosomal region (arrowhead). Bar = 10 μm.

and acrosome-reacted sperm (Fig. 2B) reflects detachment of albumin from sperm surface. Therefore, as shown in Fig. 2B, albumin extracted from the uncapacitated epididymal sperm is thought to be peripherally associated with sperm plasma membrane. Difference of protein propyles between ST and U suggests differential extraction of sperm protein happened by washings prior to detergent extraction and extraction time. Although more harsh treatment (ST) did not completely demembranated the plasma membrane overlaying the apical segment of sperm head, it is plausible that peripheral proteins associated with plasma membrane overlaying the apical segment of sperm head is thought to be greatly reduced in the ST and SW* fraction by repeated washings and detergent treatment, respectively. And albumin found in the ST and SW* may reflect remained amount in the capacitated and acrosome-reacted sperm, respectively. These results suggests that albumin constitutively binds to sperm surface during the epididymal storage and is released during the capacitation and only the lilimited amount of albumin is associated with spermatozoa after the AR. Detachment of albumin was not affected by the Ca^{2+} in the media. It has been reported that the sperm capacitation requires only micromolar concentration of Ca^{2+} in the media (Fraser, 1987). Therefore, detachment of albumin partly explains the proceeding of Ca^{2+} independent capacitation. After extraction with Triton X-100 for 20 min, most of sperm were permeabilized and WGA-FITC labeling occurred in the plasma membrane overlaying the apical segment of sperm head. It was different from the labeling observed in the uncapacitated epididymal sperm (Fig. 3A). The labeling on the principal segment was similar to that of acrosome-reacted sperm (Fig. 3B, C, and C′). It suggests that permeabilization of plasma membrane overlaying the apical segment occurred under this mild detergent treatment, and redistribution of surface antigens took place. Similar event was reported to occur after Ca^{2+} influx prior to AR (Flechon, 1985; Lee and Ahuja, 1987).

BSA-FITC weakly bound to the apical region of testicular sperm head (Fig. 4A). In contrast, extensive labeling occurred in caudal sperm surface covering the apical region and post acrosome region, middle piece and tail of acrosome intact sperm (Fig. 4B and C). Differences in labeling intensities between the testicular and epididymal sperm could be explained by surface charge. During the epididymal transit, negatively charged luminal components are associated with sperm surface (Hammerstedt *et al.*, 1979; Moore, 1979). Therefore, increase of negative charge could explain the intense labeling of epididymal sperm by BSA-FITC. Periacrosomal region of the acrosome-reacted sperm was not labeled by BSA-FITC. It suggests that inner acrosomal membrane exposed externally has low affinity for albumin after AR. Inner acrosomal membrane of acrosome-intact sperm contacts previousely with vesicular content of acrosome. Therefore, the inner acrosomal membrane might be somewhat different from plasma membrane overlaying the acrosome as regarding to biochemical properties such as intramembraneous particle distribution (Flechon, 1985; Olson and Winfrey, 1991), charge distribution (Moore, 1979), carbohydrate distrbution (Lee and Ahuja, 1987; Gye, 1994), and lipid composition (Langlais and Robert, 1985). Labeling by WGA which has the affinity to oligosaccharide chain having the terminal GlcNAc and sialic acid residues (Kobata and Yamashita, 1993) bound to apical segment of cauda epididymal sperm (Fig. 3A). On the other hand Triton X-100-treated sperm showed labeling on the principal segment, and the same labeling pattern was observed in the acrosome-reacted sperm. It suggests that Triton X-100 treatment permeabilized the plasma membrane of sperm head. Labeling on the apical segment of sperm head reflects intense glycosylation of sperm surface antigens with highly negatively charged sugar moeities such as GlcNAc and sialic acid. Negatively-charged sperm surface might permitted binding of neighboring BSA molecules to sperm surface by charge interaction. WGA labeling pattern also partly explains why the BSA had strongly bound to surface of apical segment of epididymal sperm head and decrease of the binding in the capacitated and acrosome-reacted sperm.

Detachment of albumin from the sperm surface has several physiological importances with regard to fertilization competence of sperm during capacitation, First, it results in increase of membrane fluidity. Cholesterol/phospholipid ratio increases during capacitation (Langlais and Roberts, 1985). The structure of plasma membrane is explained by the fluid mosaic model. Thus, integral or peripheral proteins of sperm plasma membrane may move within the membrane depending on membrane flexibility. In fact, movement of intramembrane particles or lateral movement of peripheral proteins occurs on sperm plasma membrane (Koehler, 1976; Flechon, 1985; Suzuki and Yanagimachi, 1986) and are dependent on membrane flexibility. These changes are regarded as prerequisite for acrosome reaction. Lipid transfer protein (LTP) which transfers cholesterol from sperm to other luminal proteins was known to promote capacitation (Ravnik *et al.*, 1992). Albumin was also known to have a lipid transfer activity (Stewart-Savage, 1993). Decrease in cholesterol content of the sperm plasma membrane is mediated by albumin (Davis, 1980; Go and Wolf, 1985). Therefore, detachment of albumin from sperm surface might be responsible for the change in lipid composition during sperm capacitation and it resultes in increase of the membrane fluidity. Second, detachment of albumin may cause unmasking of hidden antigens (Fraser, 1984). Capacitation is accompanied with acquisition of zona binding ability of sperm (Heffner and Storey, 1982), and unmasking of sperm surface antigens involving the putative ZP receptors are known to responsible for the process (Fraser *et al.*, 1990). Third, binding of albumin possibly makes the sperm surface antigens more susceptable to proteinase or glycosidase of female genital tract or sperm itself, and results in modification of antigen structure to more mature form suitable for interaction with egg and prone to AR. Recently BSA was known to stimulate phosphorylation of several tyrosine-containing proteins (Visconti *et al.*, 1995) which expected to be involved in signaling pathway culminating to acrosome reaction (Ward and Kopf, 1993).

Conclusively, Ca^{2+}–independent detachment of sperm surface-associated albumin occurs during capacitation *in vitro*, and binding of albumin to acrosome-reacted sperm was greatly reduced after AR. These changes may promote the acquisition of fertilizing competence of spermatozoa, and AR might be more feasible to happen in response to external stimuli after albumin-mediated change of plasma membrane during capacitation.

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정자의 수정능력획득 과정 동안 정자표면의 Albumin의 이탈현상

계명찬 · 김문규(한양대학교 자연과학대학 생물학과)

생쥐 정자의 수정능력 획득과정 및 첨체반응을 전후로 정자표면과 albumin의 상호작용을 조사하였다. 부정소 정자를 체외에서 배양하는 과정에서 정자표면에서 이탈된 단백질의 분석과 함께 FITC-bovine serum albumin으로 정자를 형광염색하여 정자표면에 대한 albumin의 결합양상의 변화를 조사하였다. 90분간 정자를 배양한 후 정자를 제거한 후 농축한 배양액내에 정자 또는 부정소액에서 기원한 여러종의 단백질과 함께 albumin이 다량으로 발견되었다. 정자의 체외배양 과정에서 일어나는 albumin의 이탈은 배양액내의 Ca^{2+}과 무관하게 일어났다. BSA-FITC는 정소내 정자의 두부표면에 미약하게 결합한 반면 미부부정소 정자의 첨체표면에는 다량 결합하였다. Ca^{2+}-ionophore인 A23187으로 첨체반응을 유발한 정자의 두부 표면에서는 후첨체부위만이 강하게 염색되었다. 이러한 결과는 정소 및 웅성 생식수관내에서 정자표면에 부착된 albumin이 자성 생식수관을 거치는 동안 이탈됨을 시사하며 이러한 현상은 정자의 수정능력획득과 밀접한 관련이 있는 것으로 사료된다.