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ATP-binding cassette transporters ABCA1, ABCA7, and ABCG1 in mouse spermatozoa ☆

Carlos R. Morales [a], Andrea L. Marat [a], Xiaoyan Ni [a], Yang Yu [c], Richard Oko [c], Brian T. Smith [b], W. Scott Argraves [b,*]

[a] Department of Anatomy and Cell Biology, McGill University, Montreal, Que., Canada H3A2B2
[b] Department of Cell Biology and Anatomy, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425, USA
[c] Department of Anatomy and Cell Biology, Queen's University, Kingston, Ont., Canada K7L 3N6

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ABSTRACT

Mammalian spermatozoa lose plasma membrane cholesterol during their maturation in the epididymis and during their capacitation in the female reproductive tract. While acceptors such as high-density lipoproteins (HDL) and apolipoproteins A-I (apoA-I) and J have been found in male and female reproductive tracts, transporters that mediate cholesterol efflux from plasma membranes of spermatozoa to such acceptors have not yet been defined. Candidate transporters are members of the ATP-binding cassette (ABC) transporter superfamily including ABCA1, ABCA7, ABCG1 and ABCG4, which have all been implicated in the transport of sterols and phospholipids to apolipoproteins and HDL. Here we show that mouse spermatozoa in the seminiferous tubules and epididymis express ABCA1, ABCA7 and ABCG1, but not ABCG4. Moreover, we show that ABCA1, ABCA7, and ABCG1 antibodies decrease cholesterol efflux from spermatozoa to lipid acceptors apoA-I and albumin and inhibit *in vitro* fertilization.

The loss of cholesterol from the spermatozoa plasma membrane is key to spermatozoa maturation and capacitation, which together render the spermatozoa capable of interacting with an oocyte and inducing the acrosome reaction [1–3]. In the male reproductive tract, the cholesterol acceptors apoA-I and apoJ are both secreted by the epididymis and implicated in spermatozoa maturation [4–6]. However, the significance and role of these epididymal proteins are still poorly understood. ApoA-I and apoJ are also synthesized by epithelial cells of the uterus and oviduct [7,8]. As components HDL particles, these apolipoproteins are thought to mediate association of HDL particles with spermatozoa plasma membranes and to serve as cholesterol and phospholipid acceptors in the process of lipid efflux. Lipid efflux is considered one of the key events of spermatozoa capacitation that leads to acrosome reaction and fertilization. Apolipoprotein particles enriched with spermatozoa lipids are believed to deliver the cholesterol to principal cells of the epididymis [4,5,9] and to epithelial cells of the female reproductive tract where the apolipoprotein particles can be endocytosed via endocytic receptors such as megalin/LRP-2 and cubilin [7].

Although the mechanism by which cholesterol is effluxed from spermatozoa plasma membranes to lipid acceptors is not known, ABC transporters including ABCA1, ABCA7, ABCG1, and ABCG4 represent candidates since they mediate the transport of cholesterol from other types of cells to lipid-poor apoA-I and to lipoprotein particles (e.g., HDL) [10]. Here we evaluated murine spermatozoa for the expression of ABC transporters known to support apolipoprotein-mediated cholesterol release. We also evaluated their roles in the transport of cholesterol from the sperm plasma membrane to lipid-poor apoA-I and albumin and in facilitating fertilization.

Materials and methods

Antibodies. Rabbit ABCA1 antibody was purchased from Novus Biologicals (Littleton, CO). Rabbit antibodies to ABCG1 and ABCG4 were from Alpha Diagnostics International (San Antonio, TX). Monoclonal ABCA7 antibody (KM3096) [11] was provided by Dr. Katzumitsu Ueda (Kyoto University, Japan) and Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan).

Immunocytochemistry. Testes and epididymides from CD-1 mice (Charles River, Montreal, Que.) and 7-week mice homozygous for targeted deletion of *ABCA1* [12] (provided by Dr. Yves Marcel, University of Ottawa Heart Institute) were immersed in Bouin's or in 5% paraformaldehyde. The tissues were embedded in paraffin, sectioned and immunostained with ABCA1, ABCG1, and ABCG4 antibodies as previously described [5,6]. For ABCA7 staining, sections were blocked with 2.5% horse serum in PBS prior to antibody

* Corresponding author. Fax: +1 843 792 0664.
E-mail address: argraves@musc.edu (W.S. Argraves).

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incubation. Sections were incubated for 30 min with horse anti-rabbit/mouse Biotinylated Universal Antibody (Invitrogen, Burlington, ON) and incubated with Vectastain Elite ABC Reagent (Burlingame, CA).

For confocal microscopy, the caput epididymis was removed and placed in HEPES-buffered Krebs Ringers bicarbonate (KRB-HEPES) and minced. The spermatozoa-containing supernatant was collected, centrifuged at 800g at rt for 5 min and the pellet resuspended in 2 ml PBS. An aliquot (100 μl) of the suspension was fixed on a glass slide with 3.7% formaldehyde for 10 min and blocked with 3% goat serum or with 2% horse serum for 30 min. The slides were incubated with ABCA1, ABCA7, or ABCG1 antibodies or non-immune IgG for 60 min at rt and washed with PBS. The slides were incubated with FITC-conjugated secondary antibodies followed by PBS washing. Nuclei were stained with Hoechst 33342 (Molecular Probe, Eugene, OR).

RNA isolation and RT-PCR. Testes from an adult male CD-1 mouse were removed, washed with Hank's Balanced Salt Solution (HBSS), decapsulated and minced. The seminiferous tubules were then suspended in 10 ml of HBSS containing 0.4 mg/ml collagenase, 0.664 mg/ml DNaseI, 6 mM sodium pyruvate and 2 mM sodium L-lactate and incubated at 37 °C for 10 min. Trypsin (18 mg) was added and the suspension incubated for 15 min with agitation. The supernatant, containing germ cells, was collected and subjected to centrifugation at 700*g* for 5 min. The pellet containing 90% spermatids was resuspended in HBSS and 2×10^7 cells extracted using an Oligotex RNA isolation kit (Qiagen). Total RNA was isolated from mouse neonatal brain using Trizol (Invitrogen, Carlsbad, CA). cDNA was made from total RNA (1 μg) using an iScript kit (BioRad, Hercules, CA). Primers pairs and cycling conditions used for RT-PCR are shown in Supplemental Table I.

Immunoblot analysis of spermatozoa extracts. The epididymides were separated into caput, corpus and cauda. The tissues were placed in Dulbecco's Modified Eagle Medium (Invitrogen Corporation, Burlington, ON) containing Complete Protease Inhibitor Cocktail (Roche, Palo Alto, CA), and minced. The spermatozoa-containing supernatant was collected and centrifuged at 500*g*, 4 °C for 5 min. The spermatozoa were resuspended in 100 μl of 1.0% NP40, 154 mM NaCl, 0.4 mM Tris, pH 8.0 containing protease inhibitors (Roche). After 30 min incubation, the lysate was centrifuged at 10,000*g*, 4 °C for 10 min. Aliquots (20 μg protein) were subjected to SDS–PAGE (reducing conditions) and transferred to Hybond-ECL membranes (Amersham Biosciences, Piscataway, NJ). Detection was achieved using the ECL+ Western Blotting Detection System (Amersham).

Spermatozoa cholesterol efflux assays. Caput, corpus and cauda epididymides ($n = 3$) were removed and finely chopped and transferred to 5 ml of KRB-HEPES. The homogenates were incubated at 37 °C for 10 min and the released spermatozoa pelleted by centrifugation at 500*g*, for 10 min at 25 °C. The pellets were resuspended in 2 ml of KRB-HEPES at 1×10^7 spermatozoa/ml. An aliquot (100 μl) of the cell suspension was mixed with 100 μl of 50 μg/ml human apoA-I in KRB-HEPES plus and minus antibodies to ABCA1 (10 μg IgG/ml) or ABCG1 (5 μg IgG/ml) and incubated at 37 °C and 5% CO_2 for 1 h. In control reactions, spermatozoa were mixed 1:1 with KRB-HEPES lacking apoA-I. In separate experiments, delipidated BSA was used instead of apoA-I. After 1 h incubation, spermatozoa were pelleted at 500*g* for 10 min, the supernatant saved and the pellet washed with KRB and resuspended in 140 μl of PBS. Aliquots (50 μl) of the spermatozoa suspension and supernatant were analyzed for cholesterol using an Amplex Red Cholesterol Kit (Molecular Probes).

Fertilization assays. Epididymides were in M2 capacitating medium (M2) [13] plus and minus ABC transporter antibodies (0.1 μg IgG/ml). The contents of the epididymides were squeezed out, using sterile forceps. Spermatozoa were allowed to "swim out" for 10 min at 37 °C, 5% CO_2. The spermatozoa were overlaid with 2× volume of M2 for 30 min at 37 °C, 5% CO_2 for "swim-up". The upper 1/3 of the suspension was taken and adjusted to $1–5 \times 10^6$ spermatozoa/ml for fertilization. Oocytes were isolated from 10-week CD-1 females superovulated by intraperitoneal injection of 7 IU pregnant mare serum gonadotropin (Sigma) followed 24 h later with 5.0 IU human chorionic gonadotropin (hCG, Sigma). Oocytes were collected from oviducts into antibody-containing M2 15 h after hCG injection. Cumulus oophorus cells were removed by treatment with 0.1% hyaluronidase in M2. The cumulus-free oocytes were rinsed in M2 and groups of 10 oocytes fertilized with spermatozoa at 37 °C, 5% CO_2. Fertilization was assessed 18 h later by counting the number of zygotes as compared to oocytes [14].

Statistical analysis. The Chi-square test was used to test the significance of differences between groups.

Results

ABC transporter expression in the testis

Immunohistological analysis of testicular sections revealed ABCA1 staining of the cytoplasm of Sertoli cells (Fig. 1A and C). ABCA1 was also detected on the head and tail of step 16 spermatids (stage VII–VIII of spermatogenesis) (Fig. 1A and C). By contrast, spermatogonia, spermatocytes and round spermatids (step 1–7) and elongating spermatids (step 8–15), showed no reactivity with ABCA1 antibody (Fig. 1A).

The expression of ABCA7 in testis was similar to that of ABCA1. Sertoli cells exhibited staining, as did the heads and tails of step 16 spermatids, albeit the relative level of ABCA7 staining of the tails was less than that observed for ABCA1. No ABCA7 expression was detected in spermatogonia, spermatocytes, or round spermatids (Fig. 1D).

By contrast to ABCA1 and ABCA7, only faint, intermittent ABCG1 immunolabeling was observed in Sertoli cells. While there was pronounced ABCG1 staining in the tails of step 16 spermatids, the heads were negative. No ABCG1 expression was evident in spermatogonia, spermatocytes, or round spermatids (Fig. 1E).

No ABCG4 immunoreactivity was detected in the testis. However, the ABCG4 antibody was found to react with other mouse tissues including the brain cortex in which neuronal cells were positive (data not shown). The results of histological analysis of ABC transporter expression in the testis are summarized in Supplemental Table II.

ABCA1, ABCA7, and ABCG1 transcripts were detectable in spermatid RNA preparations, however, ABCG4 RNA was not detectable. The ability of the ABCG4 primers to specifically amplify mouse ABCG4 was verified using brain cDNA. The findings obtained from RT-PCR analysis are consistent with those from immunohistological analyses (Supplemental Fig 1).

ABC transporter expression in the epididymis

Immunostaining of epididymal sections revealed ABCA1 staining of the heads and tails of spermatozoa located throughout the caput, corpus and cauda epididymis (Supplemental Fig. 2A and B). Principal cells of the caput, corpus and cauda epididymis also exhibited a pronounced cytoplasmic ABCA1 immunostaining (Supplemental Fig. 2A–C). A stronger level of staining was observed along the apical surface at the base of the microvilli (Supplemental Fig. 2A–C, arrows). The degree of ABCA1 immunostaining of principal cells was similar in all three region of the epididymis.