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INVITED RESEARCH HIGHLIGHT

Sperm Biology

Epididymosomes: a heterogeneous population of microvesicles with multiple functions in sperm maturation and storage

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Extracellular microvesicles present in the epididymal fluid have been named epididymosomes. Many epididymosome-associated proteins are transferred to spermatozoa during their maturation in the excurrent duct. Epididymosomes are heterogeneous, with their size varying between 50 and 250 nm. Two distinct population of epididymosomes characterized by different protein compositions and diameters have been isolated from the bovine epididymal fluid using different centrifugation protocols. One subpopulation of epididymosomes was characterized by CD9 and other tetraspanin partners. Transfer of proteins from these epididymosomes to maturing spermatozoa in co-incubation experiments was inhibited by antibodies against tetraspanin proteins. This suggests that this subpopulation of epididymosomes is involved in the acquisition of proteins involved in maturation by spermatozoa in the epididymis. The other population of epididymosomes was characterized by ELSPBP1 (epididymal sperm binding protein 1), known for its affinity for the phospholipid choline group. Flow cytometric analyses showed that ELSPBP1-positive epididymosomes only interacted with dying or dead epididymal spermatozoa in a Zn^{2+}-dependent manner. BLVRA (biliverdin reductase) was identified as a partner of ELSPBP1. This enzyme reduces biliverdin to bilirubin: two molecules with powerful anti-oxidant properties. We hypothesize that BLVRA is involved in an ROS-scavenging mechanism protecting live epididymal spermatozoa against detrimental molecules (ROS) released by dying cells. Therefore, it appears that there are at least two epididymosome population with distinct functions: targeting specific proteins to transiting spermatozoa by tetraspanin-mediated membrane fusion, and protection of epididymal spermatozoa against ROS released from dying cells. Further work is needed to understand functions of epididymosomes in epididymal physiology and sperm maturation and storage.

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THE EPIDIDYMIS

The epididymis into which the vasa efferentia empty is formed by a single convoluted tubule classically divided into the proximal caput, the elongated corpus, and the distal bulbous cauda epididymidis. An initial segment proximal to the caput epididymidis, characterized by specific histological features, is the hallmark of some mammalian species, particularly in rodents.[1] Epididymal functions consist of sperm transport, concentration, and storage. The spermatozoon reaching the epididymis, even though differentiated, is unable to fertilize. The epididymis is known to be involved in sperm maturation, which consists of the acquisition of forward motility properties and fertilizing ability. Epididymal sperm maturation is thus a major function of the epididymis.[2] The epididymis is the signature of a male reproductive tract of vertebrate phyla practicing internal fertilization. The reproductive strategies from one species to the other showing great variation, sperm deposition at copulation is not always synchronized with ovulation.[3] In the cat, for example, copulation induces ovulation whereas these two events occur at 4–6 months' time interval in bat species. It is thus hypothesized that the epididymis generates a heterogeneous population of male gametes. Following deposition in the female genital tract, each cohort of male gametes will be available for fertilization at different time points; increasing the fertilizing window of a given ejaculate awaiting ovulation.

The epididymis is a single, convoluted tubule measuring up to 90 m in animal stock species like bulls. The pseudostratified epididymal epithelium defining the lumen is formed by epithelial cells with very efficient tight functions.[4] Under androgenic stimulation, the epididymal epithelium secretes a complex mixture of proteins; the proteomic signature of the epididymal intraluminal milieu shows great variation along the excurrent duct. These proteins, as well as other macromolecules of the epididymal intraluminal compartment, interact with the transiting spermatozoa. The composition of the intraluminal milieu varying along the epididymis; the biochemical modifications undergone by the spermatozoa and occurring in a sequential manner along the epididymis have been defined as sperm maturation.[5]

Since the early 1980s, it has become clear that surface biochemical properties of spermatozoa undergo major modification while transiting along the epididymis. In fact, sperm surface proteins have been shown to be added, retrieved or to undergo posttranslational modifications during epididymal transit.[6,7] Many sperm proteins

secreted by the epididymal epithelium have been identified, and the functions of some of them have been described. Some of them are added to very precise surface domains of the sperm; the mechanisms underlying proteins targeting sperm surface domains remain to be described. As they can be retrieved from the sperm surface by high ionic strength solutions, sperm epididymal proteins were thought to be coating proteins retained to sperm surface by electrostatic interactions. Unexpectedly, some epididymal proteins behave as integral membrane proteins once transferred to the male gamete.[6] Others are anchored to spermatozoa by glycosylphosphatidylinositol (GPI) residues.[8] According to cell biology dogma, in order to be GPI-anchored or be integrated into the plasma membrane phospholipid bilayer, such proteins have to be associated with the endoplasmic reticulum, to undergo posttranslational modifications in the Golgi apparatus and to be integrated to a cell plasma membrane during secretory vesicle fusion with the plasma membrane. Altogether, this process constitutes the merocrine pathway of secretion. Thus, acquisition by the transiting spermatozoa of some extracellular proteins secreted by the epididymal epithelium is incompatible with the classical merocrine secretion pathway. One example of these epididymal proteins targeting a specific sperm surface domain, the acrosomal cap, is P34H (or P26h), characterized in man (and hamster). Many other proteins behaving in a similar way have been studied.[9,10]

P34H/P26h is secreted by the epididymal epithelium. While P26h is undetectable when hamster caput epididymal spermatozoa are probed with specific antibodies, this protein generates a strong signal when the same detection procedure is applied to cauda epididymal spermatozoa.[7] Other experimental evidence shows that this newly-acquired sperm protein is GPI-anchored to spermatozoa.[11] Together with the fact that the cDNA sequence encoding for P26h does not harbor the signal peptide sequence, these observations challenge the accepted mechanism of secretion.[12] Another secretory pathway has thus to be considered to explain how some proteins are acquired during the maturational process during epididymal transit.

APOCRINE SECRETION AND EPIDIDYMOSOMES

Apocrine secretion is an alternative secretory pathway; milk secretion by mammary glands being the usual example cited. The process consists of the formation of apical blebs by secretory epithelial cells. Very few organelles are found in these blebs; they are devoid of mitochondria, secretory vesicles, endoplasmic reticulum, and ribosomes. The blebs detach from the apical membrane and release their content when disintegrating in the lumen.[13,14] This process is known as apocrine secretion. Small membranous vesicles are contained in these blebs and are released into the extracellular compartment by apocrine secretion.[10,15,16] This mode of secretion is rather common in the male reproductive tract, as it has been described in the rat coagulating gland, the vas deferens, the prostate and the epididymis. As a result, a relatively high concentration of extracellular microvesicles is found in human seminal plasma, which have been named prostasomes, as they were thought to originate from the prostate.[9] Recently, it has been shown that microvesicles originating from the epididymis are also present in human seminal plasma.[17] These extracellular microvesicles originating from the epididymis have been named epididymosomes.

Epididymosomes were first described by Yanagimachi *et al*. in the mid-1980s.[18] They were described as membrane-bound vesicles of 50–250 nm that can be found in close contact with sperm plasma membrane in the intraluminal compartment of the hamster epididymis. These vesicles have now been described in different species such as mice, rats, bulls, rams, and men.[9]

Epididymosomes have been particularly studied in the bovine model. Epididymides from sexually mature bulls can easily be obtained at a low cost from the slaughterhouse, and the size of the organ allows recovery of large volumes of uncontaminated epididymal intraluminal fluid. Serial centrifugation protocols allow recovery of spermatozoa, of the fluid fraction, and of epididymosome suspensions from different epididymal segments.[19] The completed bovine genome sequencing allows … omic analyses of epididymal tissues and sub-fractions. For example, LC-MS/MS analysis of epididymosome protein content shows great variation when microvesicles prepared from fluids collected from different epididymal segments are compared.[20]

It has been hypothesized that epididymosomes delivered to the intraluminal compartment of the epididymis are involved in protein transfer to the maturing spermatozoa. When epididymosomes collected from a proximal epididymal segment are incubated *in vitro* with distally-collected spermatozoa, only a subset of proteins associated with epididymosomes is transferred to the male gamete.[21,22] We have shown that this transfer is saturable with time, and it is temperature- and pH-dependent. Interestingly, the physiological pH of epididymal intraluminal is 6.5: the optimum pH for interaction between epididymosomes and spermatozoa to occur *in vitro*. Whereas Mg^{2+} and Ca^{2+} added to the co-incubation medium have no effect on protein transfer, Zn^{2+} strongly potentiates epididymosome-spermatozoa interaction *in vitro*. The transferred proteins in co-incubation experiments are preferentially found on the sperm acrosomal cap and the mid-piece.[21]

Proteins associated with epididymosomes are compartmentalized; they can be located within the vesicle while others are externally exposed. Some surface epididymosomal proteins are segregated in raft membrane domains, others not. The way proteins are associated with epididymosomes seems to determine the sperm cell sub-domains to which they will be transferred. For example, MIF (Microphage migration Inhibitory Factor) is located within the epididymosomes and will be transferred to the intracellular dense fibers of the sperm flagellum. P26h/P34H is associated with raft membrane domains of epididymosomes.[23] It is also found in these sperm membrane sub-domains where it can play a role in cell (sperm)-extracellular matrix (zona pellucida) binding.[7,24]

The epididymal apocrine secretion pathway releases into the intraluminal compartment extracellular microvesicles named epididymosomes. *In vitro* experiments strongly support the concept that epididymosomes transfer selected proteins to spermatozoa during the epididymal maturation process. The mechanisms of the epididymosome-spermatozoon interaction underlying protein transfer remain to be elucidated.

HETEROGENEITY OF EPIDIDYMOSOMES

Epididymosomes are heterogeneous in size when observed at the electron microscopic (EM) level. Some studies reveal that these microvesicles have distinctive EM characteristics, suggesting that a different type of vesicles was found in a given epididymal fluid sample.[16] Extracellular microvesicles represent a novel mechanism of intercellular communication, and some evidence suggests that they may be involved in different pathologies. Efforts have been made to classify these vesicles according to their biophysical properties, their biochemical composition,

and their intracellular origin. The most studied vesicles are exosomes characterized by a diameter of 50–100 nm. Being enriched in cholesterol and sphingomyelin, they contain membrane-raft domains. Tetraspanin complexes are also part of the exosome signature: CD9 being used as a marker of this type of microvesicle.[25] Whereas prostasomes from human seminal fluid can be fractionated into distinct subpopulation harboring different enzymatic activities, few attempts have been made to isolate different population of microvesicles from epididymal fluid.[26] The total population of epididymosomes free of other cellular contaminants, like microsomes, has been used to understand the role of these microvesicles in sperm physiology. More recently, we have applied a different purification protocol, originally designed to purify exosomes from other biological fluids, in an attempt to fractionate epididymosomal subpopulation.

The CD9-positive epididymosomes represent the subpopulation of total epididymosomes with the smallest diameter of 10–100 nm. Co-immunoprecipitation experiments reveal that CD26 and CD224 are CD9 partners. These CD9-positive vesicles fuse with spermatozoa, and inhibitory properties of anti-DC9 and anti-C26 strongly suggest that tetraspanin complexes of these microvesicles are involved in the fusion with epididymal spermatozoa. Interestingly, P25b and GliPriL1, both known to be involved in the sperm-egg interaction, and MIF and AKR1B1, proteins involved in sperm motility, are highly enriched in CD9-positive epididymosomes compared with unfractionated epididymosomes. From consideration that sperm motility and egg recognition are two properties acquired by spermatozoa during epididymal maturation, it is highly suggestive that CD9-positive epididymosomes are involved in the acquisition of these sperm functions.[27]

Interestingly, CD9-positive epididymosomes preferentially bind to or fuse with live spermatozoa, suggesting that the epididymis "processes" only spermatozoa that will survive the transit along the male reproductive tract.[27]

The other population of epididymosomes shows a higher affinity for dead spermatozoa. These epididymosomes are enriched in ELSPBP1 (EpididymaL SPerm Binding Protein 1), a protein identified by tandem mass spectrometry that is associated with a population of ejaculated bovine spermatozoa that died in the epididymis before ejaculation. ELSPBP1 was first described as HE12 in humans and CE12 in dogs.[28–30] Orthologs have also been detected in the horse, pig, and bull. The role of this protein remains elusive, but it is characterized by Type 2-binding domains. This structure suggests that ELSPBP1 shows similarities with BSP (Binder of Sperm Proteins), the major constituent of bovine seminal vesicle secretion that binds to the choline head group of sperm membrane phospholipids. BLVRA (Biliverdin Reductase A) has been identified as a partner of ELSPBP1. BLVRA uses NADPH as a proton donor to reduce biliverdin in bilirubin; the latter then uses ROS (Reactive Oxygen Species) to regenerate biliverdin. This enzymatic loop is, in fact, a ROS-scavenging process. The binding of ELSPBP1 to BLVRA, as to spermatozoa, is dependent on Zn^{2+}, a cation found in high concentration in the epididymis (unpublished data). Interestingly, both Zn^{2+} and biliverdin are found in a bovine epididymal fluid; the highest concentrations being in the caput segment. The working hypothesis is thus that in the presence of Zn^{2+}, ELSPBP1/BLVRA binds to dying spermatozoa. The enzymatic loop involving BLVRA activity acts as a scavenger of ROS generated by dying spermatozoa and protects the surviving spermatozoa against the oxidative stress. There is thus a population of microvesicles in the epididymal lumen with protective functions against deleterious molecules generated by dying sperm cells.

CONCLUSION

During its transit along the epididymis, mammalian spermatozoa undergo major biochemical modifications collectively known as sperm maturation. By using the apocrine pathway, the epididymal epithelium secretes extracellular microvesicles named epididymosomes that are involved in these processes of sperm maturation. The epididymosome population in a given epididymal segment is heterogeneous. One population of epididymosomes is CD9-positive and fuses with spermatozoa via tetraspanin complexes. During this process, they transfer to the maturing spermatozoon P25b and GliPrL1L1, necessary for sperm-egg interaction, and MIF, which modulates sperm flagellar beating. Another population of epididymosomes binds to dying spermatozoa through an ELSPBP1/BLVRA complex that is proposed to play a role in live spermatozoa protection against ROS generated by dying spermatozoa.

Epididymosomes are extracellular microvesicles secreted into the epididymal lumen playing multiple functions in sperm physiology. Additional work is needed to appreciate fully the multiple functions played by these extracellular microvesicles in sperm maturation and protection, and to understand the complex physiology of the epididymis.

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