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Immunohistochemical localization of spermadhesin AWN in the porcine male genital tract

F. Sinowatz[1], W. Amselgruber[1], E. Töpfer-Petersen[2], J.J. Calvete[2], L. Sanz[2], J. Plendl[1]

[1] Lehrstuhl für Tieranatomie II, Universität München, Veterinärstrasse 13, D-80539 München, Germany
[2] Institut für Reproduktionsmedizin, Tierärztliche Hochschule Hannover, Hannover, Germany

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Abstract. Boar spermadhesin (AWN) is a 14-kDa multifunctional protein, attached to the surface of the spermatozoa and involved in sperm capacitation and zona pellucida binding. The cellular origin of AWN was previously unknown. Moreover, the region of the male genital tract in which AWN becomes attached to the surface of spermatozoa was also uncertain. By using monospecific polyclonal antibodies against AWN, the immunohistochemical distribution pattern of AWN epitopes has been investigated in tissue sections of the porcine male genital tract. Our study has revealed that AWN is synthesized in the rete testis and in the epithelium of the seminal vesicles. The latter are also the major contributors of seminal plasma AWN. In addition, immunoblotting analysis has shown that AWN is present on epididymal spermatozoa. Our results indicate that the cellular origin of spermadhesins is species-specific. The attachment of AWN to epididymal spermatozoa is probably important in developing the capacity for fertilization.

Key words: Immunohistochemistry – Zona pellucida-binding protein – Boar spermadhesin – Pig

Introduction

Gamete recognition and binding, a key event in mammalian species-specific fertilization, is mediated by complementary molecules located on the external surfaces of spermatozoa and the oocyte. The sperm receptor of the ovum resides on oligosaccharide moieties of the glycoproteins of the zona pellucida (ZP). The sperm-associated primary ZP-recognizing molecules are carbohydrate-binding proteins (for recent reviews, see: Wassarman 1991; Calvete et al. 1992). A variety of putative sperm-associated ZP-binding proteins, including lectins and enzymes, has been identified in different mammalian species (O'Rand 1988; Sinowatz et al. 1988; 1989; Töpfer-Petersen et al. 1995). It has been shown that species-specific fertilization requires a multiplicity of molecules and therefore comparison between different species is difficult.

We have identified and characterized several low-molecular-mass (12–14 kDa) ZP-binding proteins in pig sperm. They are called AQN-1, AQN-3, and AWN, and belong to a family of carbohydrate-binding proteins, the spermadhesins (Sanz et al. 1991; 1992a-c). Spermadhesins are synthesized in the sexual accessory glands and become attached to the plasma membrane overlying the acrosomal cap of the sperm (Calvete et al. 1992, 1993).

Although spermadhesins can be isolated in large amounts from seminal plasma, their cellular origin is unknown. The aim of this study has been to examine the immunohistochemical localization of boar spermadhesin AWN in the porcine male genital tract to get detailled information about the site of its production.

Materials and methods

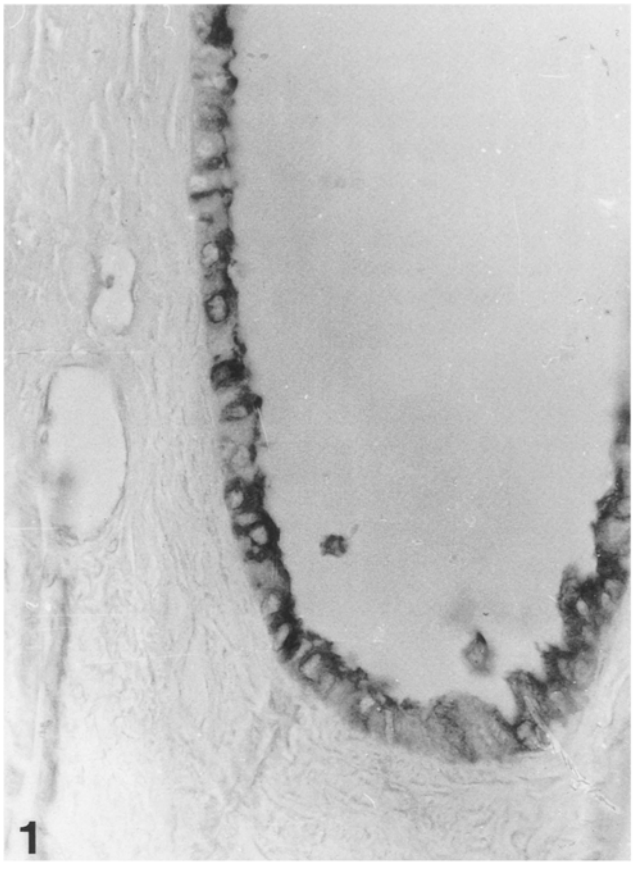
Sample preparation

Epididymal spermatozoa and seminal vesicle fluid were released from the epididymal duct and seminal vesicles of boars killed at a local slaughterhouse. Tubules were cut at different sites. Sperm were separated from epididymal fluid by centrifugation at 160 *g* for 5 min at room temperature, washed twice in 20 mM phosphate, 135 mM NaCl, pH 7.4 (PBS buffer), and resuspended in this buffer.

Freshly ejaculated sperm were collected using an artificial vagina. They were separated from seminal plasma by centrifugation in PBS buffer (160 *g* for 5 min at room temperature) and extracted overnight at 4°C with five (pellet) volumes of 2.5% (v/v) acetic acid, 10% glycerol, while being stirred gently. After centrifugation at 14 000 *g* for 15 min at room temperature, the clear supernatant was dialyzed against deionized water and lyophilized.

Isolation of AWN (isoform 1) was performed as described elsewhere (Jonàkovà et al. 1991; Sanz et al. 1992c). Polyclonal monospecific AWN antibodies, recognizing both isoforms of AWN, were raised in chicken. The IgG fraction was purified from egg yolk as described (Lösch et al. 1986), dialyzed against 20 mM

Correspondence to: F. Sinowatz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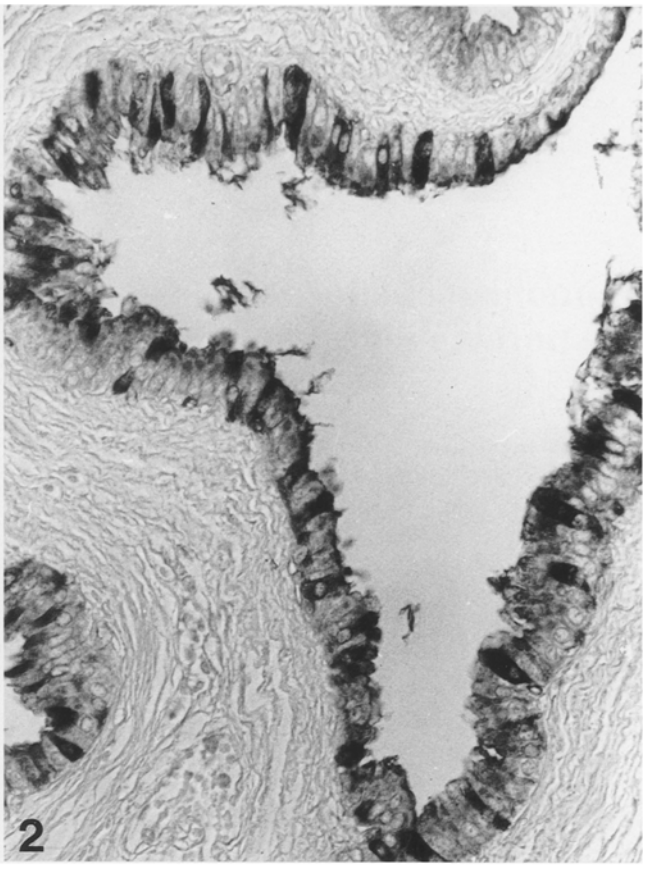
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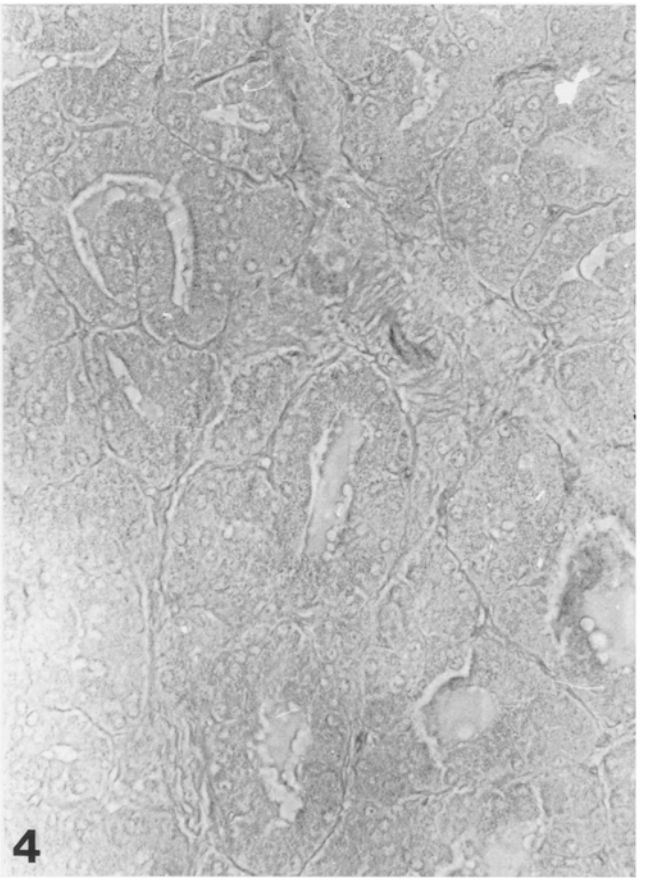
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phosphate, 135 mM NaCl, 1 mM NaN_3, pH 7.4, and stored at –70°C until used. The working dilution, defined as the dilution causing 95% maximal antibody binding to 1 µg immobilized AWN-1, was determined using a standard enzyme-linked immunosorbent assay.

SDS polyacrylamide gel electrophoresis and immunoblotting

SDS polyacrylamide gel electrophoresis was performed according to Laemmli (1970) with 7%-15% acrylamide gradient gels. Electroblotting onto nitrocellulose sheets was carried out according to standard methods (Towbin et al. 1979). The blots were blocked with 20 mM TRIS/HCl, pH 7.4, 150 mM NaCl, 5% (w/v) bovine serum albumin (TBS-BSA buffer). They were incubated for 1 h at 37°C with AWN-1 antibody (1:1000). Subsequently, they were washed with TBS-BSA buffer and incubated for 1 h at 37°C in biotinylated rabbit anti-chicken IgG (1:300). After extensive washes in TBS-BSA buffer, the blots were incubated for 1 h at 37°C with streptavidin-peroxidase (1:1000), washed with 20 mM TRIS/HCl, 500 mM NaCl, pH 7.4, and finally developed in this buffer containing 20% methanol, 1 mg/ml 4-chloro-1-naphthol (BioRad, Munich, Germany), and 15 µl H_2O_2.

Immunohistochemichal localization of AWN epitopes

Samples of tissues from testis, epididymis, ampulla ductus deferentis, prostate, bulbourethral glands, and seminal vesicles were obtained from boars recently sacrificed at a local slaughterhouse.

The tissue was fixed in Bouin's solution or in methanol-acetic acid for 3 h. Samples were dehydrated in a graded series of ethanol and embedded in paraffin. Sections (5 µm thick) were cut on a Leitz microtome and stained with hematoxylin-eosin. The localization of AWN epitopes was carried out as follows: Tissue sections were deparaffinized. Endogenous peroxidase activity was eliminated by incubation in 0.5% H_2O_2in PBS buffer for 15 min at 20°C. Non-specific protein binding was eliminated by incubation in 10% normal goat serum for 1 h at 20°C. Samples were incubated overnight at 4°C in polyclonal chicken AWN antibody (diluted 1:500 to 1:2000). Samples were washed three times in PBS buffer and once in PBS buffer with 1% BSA; a subsequent incubation in biotinylated mouse monoclonal anti-chicken IgG (1:300) in PBS-1% BSA was performed for 2 h at 20°C. After the slides were washed in PBS, the sections were incubated in streptavidin-peroxidase in PBS-BSA (1:150) for 1 h at 20°C, followed by washing. Samples were developed for 5 minutes in 50 µg/ml diaminobenzidine in PBS containing 1% (v/v) H_2O_2. All incubations were performed in a humidified chamber. The samples were left unstained or were counterstained with Mayer's hematoxylin solution, dehydrated and mounted in DePeX. The following controls were performed: 1) omission of the primary antibody, 2) replacement of AWN antibodies with normal mouse serum (1:5 to 1:100); 3) preabsorption of the primary antibody with AWN-1 in a siliconized polypropylene tube.

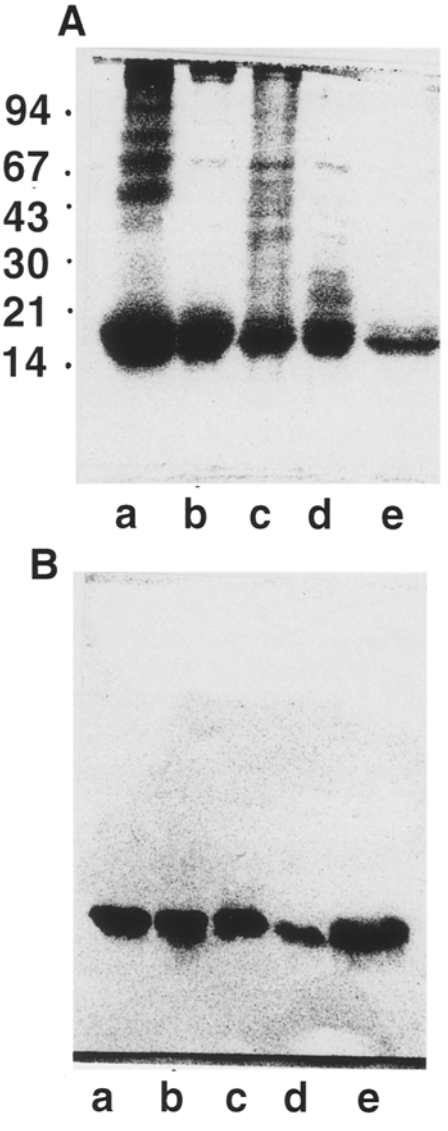

Fig. 5. A SDS- (7%-15%) polyacrylamide gel electrophoretic analysis of seminal vesicle fluid (*lane a*), seminal plasma (*lane b*), acidic extract of epididymal sperm (*lane c*), acidic extract of ejaculated spermatozoa (*lane d*), and isolated AWN-1 (*lane e*). The *numbers at the left* indicate the position of molecular mass standards (in kDa). From *top to bottom*: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme. **B** Immunoblotting analysis. Detection of anti-AWN binding activity in *a* replicate of the gel shown in **A**

Fig. 1. Testis, pig. The epithelium of the tubuli recti and the rete testis are labeled distinctly with Anti-AWN. ×320

Fig. 2. Seminal vesicles, pig. In the accessory sex glands, pronounced differences in AWN immunoreactivity are observed. Distinct labeling for immunoreactive AWN can be found in the epithelium of the seminal vesicles. ×320

Fig. 3. Epididymis, pig. Immunoreactivity for AWN is present in the stereocilia of the ductus epididymidis and the luminal content of the duct. ×320

Fig. 4. Prostate, pig. No immunostaining is seen in the prostate. ×320

Results

A characteristic labeling pattern for immunoreactive AWN was found in the different parts of the boar genital tract. In the testis (Fig. 1), the epithelium of the rete testis was clearly labeled. The germinal epithelium of the tubuli seminiferi contorti and interstitial tissue were immunonegative. In the epididymis (Fig. 3) immunoreac-

tivity for AWN was confined to the stereocilia of the ductus epididymidis. The luminal content of the duct also showed a pronounced staining for AWN. Smooth muscle cells of the epididymal duct and the interstitial tissue of the epididymis did not display immunoreactivity for AWN.

In the accessory sex glands, pronounced differences in AWN immunoreactivity were observed. Distinct labeling could be found in the epithelium of the seminal vesicles (Fig. 2). Groups of secretory cells with strong immunoreactivity for AWN in their cytoplasm were separated by weakly staining cells. The number of strongly immunopositive cells varied in the different acini of the gland. AWN immunopositive material could be seen in the lumina of the acini. The stroma of the seminal vesicles showed no immunoreactivity. In contrast to the distinct labeling in the seminal vesicles, no staining was seen in the prostate (Fig. 4) or bulbourethral glands. It can be concluded that most of the AWN activity in seminal plasma results due to secretion of the seminal vesicles.

SDS gel electrophoresis and immunoblot analysis (Fig. 5) demonstrated the presence of AWN epitopes in seminal vesicle fluid, the seminal plasma and acidic extracts of epididymal sperm. Acidic extracts of ejaculated spermatozoa were also positive for AWN.

Discussion

Whereas the molecular structure of several spermadhesins has been characterized extensively (Sanz et al. 1991, 1992a-c), there is little information about their cellular origin. In this paper, we present immunohistochemical evidence that the boar spermadhesin AWN is produced in the rete testis and in the seminal vesicles. In addition, immunoblotting analysis has shown that AWN is a major protein of the epididymal sperm extract. We therefore assume that AWN which is secreted by the epithelial cells of the rete testis is transported to the epididymis. There, it is absorbed onto the stereocilia of the ductus epididymidis and onto the plasma membrane of the epididymal spermatozoa, and may play a role in epididymal sperm maturation. It has been known for a long time that mammalian spermatozoa are released into the lumen of the seminiferous tubules in an immature state (Chang 1951; Yanagimachi 1988). Spermatozoa acquire the capacity for fertilization during passage through the epididymal duct (Austin 1951; Shivaji et al. 1990). In the boar, a large proportion of the spermatozoa appear fertile when they reach the distal caput and the proximal corpus epididymidis. The mechanisms involved in epididymal sperm maturation are poorly understood, but it seems plausible that molecules such as AWN, which occur in high concentrations in the epididymal fluid and are absorbed onto the surface of the sperm during their epididymal passage, may be involved in this process.

Most of the AWN that occurs in the ejaculate is synthesized and secreted by the epithelium of the seminal vesicles. Our previous studies have indicated that the concentration of AWN in the seminal plasma is 2 mg/ml (Dostàlova et al. 1994). Immunoblots performed by us show that acidic extracts of ejaculated spermatozoa display distinct immunoreactivity for AWN. The surface of sperm is coated with large amounts of different spermadhesins (Dostàlova et al. 1994) but the relative amounts of the different spermadhesins on the surface of ejaculated spermatozoa do not reflect their relative amounts in the seminal plasma. Thus,the amount of the spermadhesins AWN, AQN-1, and AQN-3 is increased 2-, 1.5- and 1.5-fold on sperm compared with seminal plasma, whereas the concentration of AQN-2 is 1.5 times lower in ejaculated sperm than in seminal plasma. The reason for this uneven coating of different spermadhesins on the sperm surface is not clear. Further detailed investigations are necessary to determine whether individual spermadhesins adhere preferentially to different domains on the sperm surface.

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