The receptor for urokinase-type plasminogen activator is expressed during mouse spermatogenesis

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Abstract Urokinase-type plasminogen activator (uPA) binds to a plasma membrane receptor (uPAR) that localizes plasmin generation to the cell environment. Mouse spermatozoa have surface-bound uPA, which appears to be acquired from genital tract secretions at ejaculation. We determined the presence of uPAR mRNA in spermatogenic cells and their uPA-binding activity. Northern blot and in situ hybridization demonstrated the presence of uPAR mRNA in germ cells. Binding of uPA, but not of a mutant enzyme lacking the receptor-binding domain, indicated the presence of uPAR on spermatids and spermatozoa. The uPAR and/or receptor-bound uPA may be involved in spermatogenesis, spermatozoa maturation or fertilization.

Key words: uPA receptor; Germ cell; Spermatozoa; Local proteolysis

1. Introduction

After their release from the seminiferous tubules of the testis, spermatozoa progress through the epididymis where they become mature and capable of fertilizing ova [1]. Epididymal secretions are thought to provide an optimal environment for spermatozoa maturation [2,3], a process that involves modifications of cell surface molecules. Alterations of cell surface molecules also occur in an additional maturation step, termed capacitation, that takes place in the female genital tract. It has been well established that limited proteolysis plays a role in spermatozoa maturation [4] and capacitation [5,6].

Plasmin is a tryptic protease that is thought to play an important role in extracellular proteolysis [7]. It is generated from plasminogen, by limited proteolysis catalyzed by plasminogen activators (tissue-type plasminogen activator, tPA, or urokinase-type plasminogen activator, uPA). Binding of uPA to a high-affinity, specific plasma membrane receptor, uPAR, that is present on a variety of different cell types [8,9] localizes the generation of plasmin to the close environment of the cell surface.

Previous studies have indicated that ejaculated mouse spermatozoa have cell-associated uPA. The enzyme was shown to be synthesized by epithelial cells from the caudal part of the epididymis and the vas deferens [10], and the binding of exogenous uPA to spermatozoa prepared from the vas deferens suggested the presence of uPAR [10]. However, because of difficulties in preparing spermatozoa that had not been exposed to epididymal or vas deferens secretions, the evidence for the presence of uPAR on spermatozoa was not unambiguous. We have thus re-investigated this issue, taking advantage of the availability of mice lacking uPA [11] and of molecular probes for murine uPAR mRNA [12]. Our results provide conclusive evidence that uPAR is synthesized by germ cells during spermatogenesis, and that it is present on spermatids and mature mouse spermatozoa. Together with the previously described presence of plasminogen-binding sites on these cells [13], these observations raise the question of the possible role(s) of the uPA-plasminogen system in spermatogenesis, spermatozoa maturation and/or fertilization.

2. Materials and methods

2.1. Materials

uPA-deficient mice were kindly provided by Dr. Carmeliet (Center for Molecular and Vascular Biology, University of Leuven, Belgium). CBAJ mice were obtained from IFFA CREDO (France). Conditioned media from cultures of transfected human WISH cells containing murine uPA or ΔG-uPA (a mutant form of uPA lacking the Asn^{11}–Asp^{46} receptor-binding domain of the enzyme [14] were kindly provided by Dr. Belin (Department of Pathology, University of Geneva Medical School, Switzerland). A murine uPA receptor probe (MMU-PAR1, [12]) was kindly provided by Dr. Solberg (The Finsen Laboratory, Copenhagen, Denmark). Digoxigenin-labeling reagents, *Eco*R V, *Xba*I and RNA polymerase T3, T7 were from Boehringer Mannheim (Germany). Collagenase (type IV), hyaluronidase were from Sigma (Switzerland). DMEM and FCS were from Gibco BRL (Switzerland). RNAzol was from AMS (Switzerland).

2.2. Sertoli-germ cell cultures and fibroblasts

The testes of 2-month-old uPA −/− mouse were dissected free of tunica albuginea and incubated in 5 ml of collagenase (0.1 mg/ml in PBS) at 37°C with agitation until the seminiferous tubules had become completely dissociated. The tubules were sedimented by gravity and the released cells (predominantly interstitial cells) were removed, placed in DMEM+10% FCS and used as fibroblast cultures for in situ hybridization. To remove the peritubular cells, the tubules were further incubated for 10 min at 37°C in fresh collagenase supplemented with hyaluronidase (0.1 mg/ml). Residual extratubular cells were eliminated by suspending the tubules in 1 M glycine (in PBS) until the tubules had sedimented. After being washed in PBS, the tubules were placed in DMEM and cut with fine scissors to yield ~1 mm length fragments. The tubule fragments were seeded at a density of ~100 pieces per 25-mm culture dish and cultured overnight in DMEM containing 0.5% FCS and antibiotics, in an atmosphere of 5% CO_2 in air. The cultures were then washed with PBS and further incubated in DMEM alone. After 48 h of incubation, most of the tubules had opened, with Sertoli cells attaching to the bottom of the culture dish and germ cells residing on the Sertoli cells. These cultures were termed SGCC (Sertoli-germ cell co-cultures) and used for mRNA localization and for ligand binding.

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Abbreviations: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; tPA, tissue-type plasminogen activator; ΔG-uPA, receptor-binding domain-deleted uPA; FCS, fetal calf serum; SGCC, Sertoli-germ cells co-culture; SDS-PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; NBT, 4-nitro blue tetrazolium

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2.3. Collection of germ cells and spermatozoa

Germ cells released from 48-h SGCC were collected and filtered to remove cell aggregates (containing unattached Sertoli cells); they were washed in PBS and used for ligand binding and for RNA extraction. Caudal epididymides from 2-month-old uPA −/− or wild-type (CBAJ) mice were minced in DMEM and the spermatozoa were collected after 45 min incubation (5% CO_2, 37°C) and filtered to remove epididymal tissues. The contaminating macrophages (which express uPAR) were removed by selective attachment on Petri dishes during a further incubation (30 min, 37°C) in DMEM containing 10% FCS. The spermatozoa were suspended in a 15-ml tube and the swim-up population was collected. After having been washed in PBS, the spermatozoa were used for ligand binding.

2.4. Ligand-binding and zymography

SGCC were washed 3 times with PBS, incubated in DMEM supplemented with 10% (v/v) of mouse uPA- or ΔG-uPA-rich medium for 45 min at 37°C and washed in PBS. The cells were lysed in 1 ml of 1×SDS-PAGE sample buffer (SB). Spermatozoa were similarly processed and lysed at $\sim 2\times10^6$/ml of SB. The lysates were centrifuged (10000 rpm, 4°C, 10 min) and the supernatants were analyzed by zymography [16]. Briefly, 40 μl samples were resolved by SDS-PAGE. After being washed in 2.5% Triton X-100 to remove SDS, the electrophoretic gel was placed on an indicator gel containing casein, agarose and plasminogen. PA enzymatic activity was revealed by plasmin-mediated casein lysis; zones of lysis were visualized by dark-field illumination.

2.5. In situ zymography

Germ cells and spermatozoa from uPA−/− mice were incubated with uPA- or ΔG-uPA-containing medium as above. Washed cells were mixed with the indicator gel for zymography (50 μl of cell suspension in 450 μl of indicator gel). Aliquots (70 μl) of the mixtures were applied on slides, covered with a coverslip and incubated at 37°C in a moist chamber. Lysis was visualized in a phase contrast microscope.

2.6. Northern blots

Total RNA was extracted from germ cells using RNAzol, and 15 μg samples were subjected to Northern analysis. RNA from macrophages (5 μg) was used as positive control. A ^{32}P-labeled full-length (1447 nt) antisense probe was transcribed from the linearized MMMUPAR1 construct.

2.7. In situ hybridization

Digoxigenin-labeled full-length cRNA was transcribed from linearised MMMUPAR1 to generate sense (*Eco*RV; T7) or antisense (*Xba*I; T3) probes, using a digoxigenin-labelling kit, and fragmented by alkali hydrolysis. The SGCC and 72-h culture of fibroblasts were processed for in situ hybridization and the hybridized probe was detected as described [15]

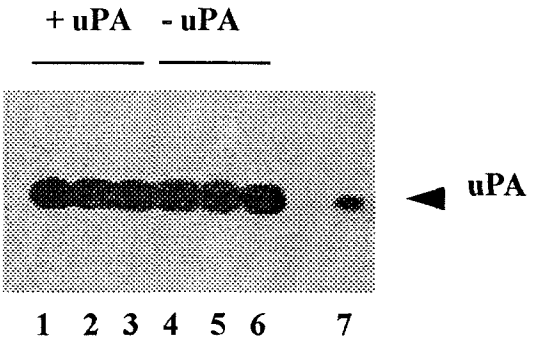

Fig. 1. Mature mouse spermatozoa have uPA activity. Spermatozoa from minced epididymides of adult CBAJ mice were incubated with (lanes 1–3) or without (lanes 4–6) uPA-containing conditioned medium [14]. Lysates of washed spermatozoa were analyzed by zymography. Spermatozoa-associated uPA activity was not increased by incubation in the presence of exogenous uPA, suggesting that uPA-binding sites had been saturated by endogenous uPA. In lane 7, an aliquot of uPA-containing conditioned medium was used as a standard.

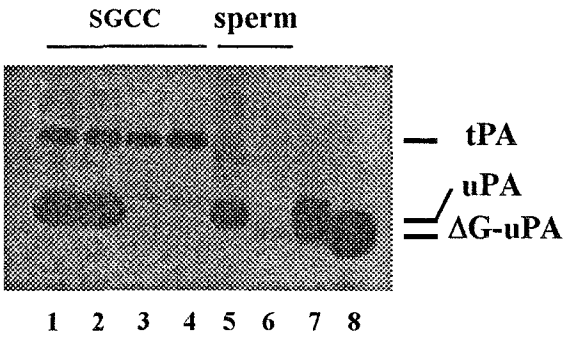

Fig. 2. Binding of uPA to SGCC and spermatozoa. Sertoli-germ cell co-cultures (SGCC) (lanes 1–4) and spermatozoa (lanes 5 and 6) from uPA −/− mice were incubated with uPA- (lanes 1, 2 and 5) or ΔG-uPA- (lanes 3, 4 and 6) containing medium, washed, lysed and analyzed by zymography. uPA activity was detected in the lysates when the cells had been incubated with uPA, but not when ΔG-uPA was used as ligand. Equivalent volumes of uPA- (lane 7) and ΔG-uPA- (lane 8) containing medium were electrophoresed in parallel, to show that the cells had been incubated in the presence of equivalent levels of uPA enzymatic activity; note that ΔG-uPA migrates slightly faster than uPA, because it lacks the receptor-binding growth factor-like domain of the enzyme.

3. Results

3.1. Germ cells and spermatozoa have uPA receptor

It has been shown previously that mouse spermatozoa express uPA activity [10]. Since ejaculated spermatozoa have more uPA than do spermatozoa obtained by spontaneous drainage of the vas deferens, it was proposed that the enzyme synthesized by the epithelial cells from the caudal epididymis and the vas deferens is secreted at the time of ejaculation and binds to a putative uPAR on spermatozoa [10]. Spermatozoa collected from minced epididymis of wild-type mice also expressed uPA (Fig. 1, lanes 4–6), and incubation in the presence of an excess of uPA did not increase their uPA activity (Fig. 1, lanes 1–3), suggesting that the putative uPAR on spermatozoa is saturated by endogenous uPA released, from reproductive tract epithelial cells or from stromal cells, during processing of the tissue.

To unambiguously demonstrate the presence of uPAR on germ cells, we took advantage of mice genetically deficient in uPA (uPA−/−). In these mice, a putative uPAR on germ cells from should be unoccupied, and it should therefore be possible to demonstrate its presence by the addition of exogenous ligand. Germ cells and spermatozoa were prepared from uPA−/− mice, and incubated in the presence of murine uPA or of a mutant form of the enzyme lacking the receptor-binding domain of the protein (ΔG-uPA). Wild-type uPA and ΔG-uPA have similar enzymatic activity, and both can be detected by zymography. In SGCC incubated in presence of uPA (Fig. 2, lanes 1 and 2), both tPA and uPA were detected, while only tPA was detected in cultures exposed to ΔG-uPA (Fig. 2, lanes 3 and 4). The tPA present in SGCC is derived from Sertoli cells [17–19]. Spermatozoa from uPA−/− mice incubated in the presence of uPA (Fig. 2, lane 5), but not of ΔG-uPA (Fig. 2, lane 6), were also found to have bound the enzyme. Zymographic analysis of equivalent volumes of the culture media used for binding demonstrated that comparable

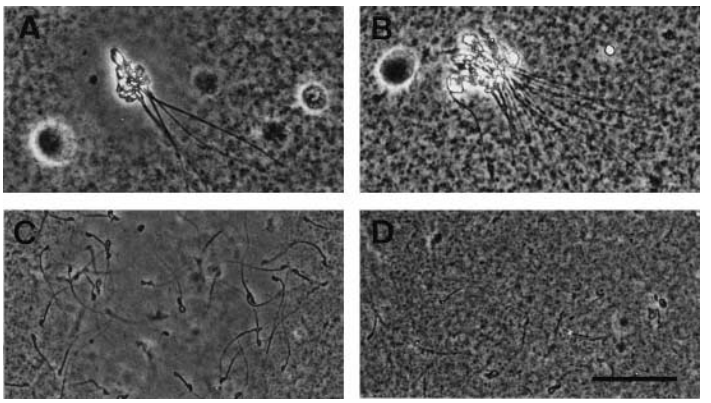

Fig. 3. Binding of uPA to testicular (A,B) and epididymal (C,D) spermatozoa. Spermatozoa from uPA−/− mice were incubated with uPA- (A,C) or ΔG-uPA- (B,D) containing medium, washed and embedded in the indicator gel for zymography. Lysis developed in the vicinity of spermatozoa when the cells had been incubated with uPA; no lysis was observed when the cells had been incubated with ΔG-uPA (B,D). Bar = 100 μm (A,B); 200 μm (C,D).

amounts of uPA and ΔG-uPA catalytic activity had been added to the SSGC and spermatozoa (Fig. 2, lanes 7 and 8). The binding of uPA, but not of the ΔG-uPA mutant protein, suggests the presence of the uPAR in SGCC mixed cultures and, as proposed previously [10], on spermatozoa.

3.2. Expression of surface uPAR during spermatogenesis

Binding experiments using SGCC do not indicate at which stage germ cells start to express functional uPAR. To resolve this issue, in situ zymography was performed. Germ cells, including spermatocytes, spermatids, testicular spermatozoa and epididymal spermatozoa were obtained from uPA−/− mice, and incubated in the presence of uPA or ΔG-uPA. The cells were then washed and embedded in a substrate layer in the presence of plasminogen. Proteolytic plaques developed around cells that had been incubated with uPA, but were not observed around cells incubated with the ΔG-uPA mutant enzyme (Fig. 3). No evidence for binding of uPA was obtained in the case of spermatocytes (data not shown), while uPA activity was revealed around spermatids (data not shown) and spermatozoa, indicating the presence of uPAR on these cells.

3.3. uPAR mRNA in germ cells

To further document the expression of the uPAR in germ cells, the presence of uPAR mRNA was explored by Northern blot analysis of total RNA isolated from preparations enriched in germ cells. A single hybridizing species with the expected size of approximately 1.5 kb, co-migrating with uPAR mRNA from mouse macrophages, was detected in total RNA from germ cells-enriched preparations (Fig. 4). mRNA localization studies were performed by in situ hybridization on cultured seminiferous tubules, using digoxigenin-labelled cRNA probes. The antisense probe revealed a positive staining reaction associated with late spermatids (morphologically recognized as flame-like germ cells), while Sertoli cells were negative (Fig. 5A), indicating that cultured Sertoli cells do not express uPAR. The specificity of the hybridization was confirmed by the use of a corresponding sense probe, which did not yield any positive cells (Fig. 5B). Further evidence for the specificity of uPAR mRNA detection was obtained using fibroblasts (which express uPAR); cytoplasmic staining was obtained with the antisense probe (Fig. 5C), but not with the sense probe (Fig. 5D). Taken together, these observations indicate that mouse germ cells contain and express uPAR mRNA, starting from the spermatid stage.

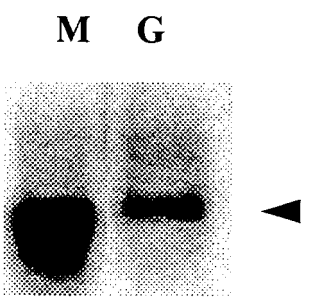

Fig. 4. Northern blot analysis of uPAR mRNA. A Northern blot containing total RNA from germ cell-enriched preparations (G, 15 μg) and from mouse peritoneal macrophages (M, 5 μg) was probed with a full-length uPAR cRNA. A single hybridizing species of ~1.5 kb was revealed in both germ cells and macrophages.

4. Discussion

Taking advantage of the availability of mice made genetically deficient in uPA, we have obtained direct evidence for the expression of a functional uPA-binding site on mouse spermatids and spermatozoa. The fact that binding requires the receptor-binding domain of the enzyme suggests that it is mediated by the uPAR; the presence of uPAR mRNA in spermatids supports this hypothesis. Attempts to localize uPAR mRNA on histological sections of testicular tissue were unsuccessful. By contrast, uPAR mRNA could be localized in cultured germ cells. This is probably related to the higher sensitivity of the latter approach, but could also be due to accumulation of uPAR mRNA in culture. In this context, the identification of an RNA-binding protein involved in degradation of uPAR mRNA [20] suggests that the stability of this transcript may vary under different conditions. In any event, the expression of uPAR on spermatozoa, which are transcriptionally silent, supports the notion that uPAR mRNA must be present in germ cells in vivo also.

The uPAR is believed to play a role in the control of extracellular proteolysis by localizing uPA-mediated plasminogen activation on or near the cell surface. The polarized dis-

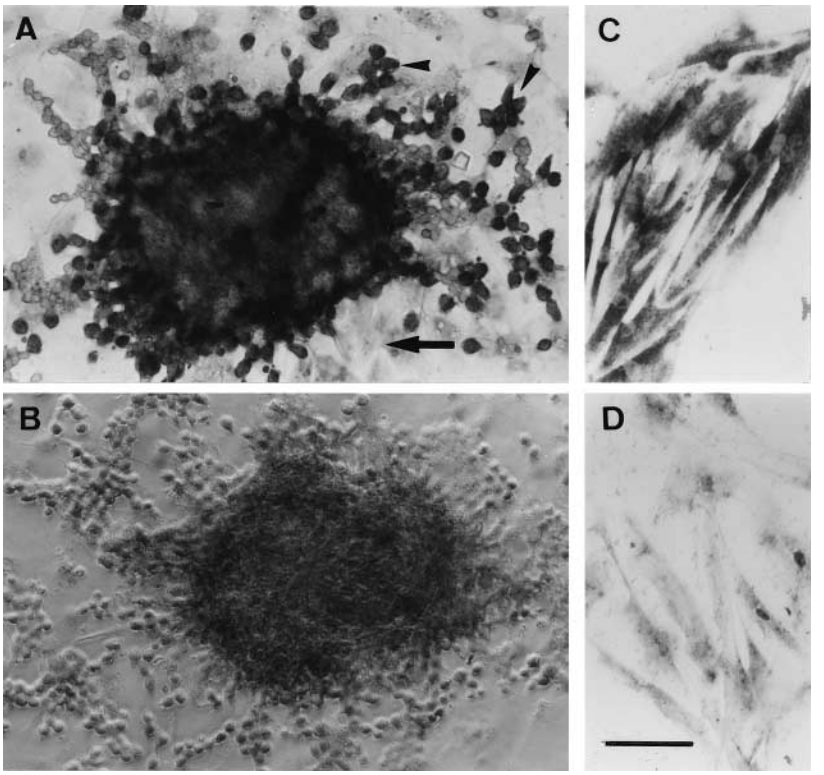

Fig. 5. In situ hybridization of uPAR mRNA. Seminiferous tubules (48 h cultures) (A,B) and cultured mouse fibroblasts (C,D) were hybridized with digoxigenin-labeled antisense (A,C) or sense (B,D) murine uPAR cRNA probes. The hybridized probe was detected by alkaline phosphatase activity, using BCIP and NBT as chromogen. Positive staining (black) was present, using the antisense probe, in germ cells (arrow head), while no signal was detected on Sertoli cells (arrow, A); the sense probe did not yield any signal (B). As a positive control for uPAR mRNA, cytoplasmic staining was revealed in mouse fibroblasts using the antisense probe (C), but not the sense probe (D). Bar = 100 μm.

tribution of the uPAR may help focus plasmin-mediated proteolysis to the leading edge of migrating cells, and thereby contribute to their directional movement [21]. In this context, the translocation of differentiating germ cells in the spermatogenic epithelium and/or their release from the epithelium into the lumen of seminiferous tubules may also be facilitated by expression of the uPAR. This suggestion is supported by the finding that plasminogen [22] and uPA [17–19] are expressed in seminiferous tubules. Alternatively, the uPAR may remain unoccupied until, presumably at the time of ejaculation, uPA is secreted by epithelial cells of the epididymis and the vas deferens [10]. In this case, the role of the uPAR and of uPA in spermatozoa physiology may be in post-ejaculation events. The capacitation of ejaculated spermatozoa involves proteolytic modifications of their cell surface [5,6]. Extracellular proteolysis could also facilitate the migration of spermatozoa towards the ampula, by preventing their adhesion to fibrin deposits on the tubal mucosa [23]. The binding of plasminogen to mouse spermatozoa and its positive effect on in vitro fertilization of mouse gametes [13] and the presence of plasminogen in the uterine lumen [24] suggest that uPAR-bound uPA could be involved in fertilization. Taken together, these observations raise the possibility that the uPAR may play a role at one or more step(s) from spermatogenesis to fertilization. Although mice deficient in either uPA [11] uPAR [25] or plasminogen [26] are fertile, the efficiency of fertilization in these mice remains to be documented. A positive correlation between spermatozoa-bound uPA activity and the outcome of in vitro fertilization in human patients [27] supports the notion of a role for the uPAR in sperm biology.

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