Bactericidal/permeability-increasing protein originates in both the testis and the epididymis and localizes in mouse spermatozoa

Zhong-Ping Zhou1,2, Xiao-Yu Xia1,2, Qiang-Su Guo1,2, Chen Xu1,2

Bactericidal/permeability–increasing protein (BPI) is an endogenous antibiotic protein with activity against gram–negative bacteria. In the present study, we examined the expression of BPI in postnatal mouse testes and epididymides as well as the subcellular localization within epididymal spermatozoa. Our results showed that, BPI mRNA was expressed in testis and epididymis independently. Throughout the epididymis, the BPI protein level gradually decreased in the epididymal epithelium in a spatial manner, specialized within the cytoplasm of clear cells in the cauda part. We detected BPI proteins in intact acrosome, implying its testicular origin; on the other hand, after the acrosome reaction, BPI proteins were observed dispersed across the entire sperm head, especially enriched at the equatorial segment. Our findings suggested a dual origin of the BPI that generated both in the testis and epididymis, and associated with mouse spermatozoa. BPI protein might be involved in the dynamics modification of the sperm plasma membrane and also the fertilization process.

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INTRODUCTION
Bactericidal/permeability-increasing protein (BPI) is a 55–60 kDa single-chain cationic protein that belongs to a conserved family of lipid-transfer proteins. BPI was originally found in neutrophil azurophilic granules1 and was later detected on the surface of neutrophils and monocytes.2,3 BPI was verified to be a product of mucosal epithelial cells as well.4 BPI exhibits powerful antimicrobial potency and selectivity against gram-negative bacteria,4 partly due to its high-affinity binding to the lipid A/inner core region of endotoxin.5 Consequently, BPI could inhibit all the pro-inflammatory activities of lipopolysaccharides (LPS), including neutrophil oxidase enzyme activation, cytokine release and nitric oxide formation.6 Members of the defensin and cathelicidin antimicrobial peptide families synergistically enhance the antibacterial activity of BPI.7 In addition to its well-documented anti-infective properties, various other BPI bioactivities have been confirmed, for instance, inhibition of the migration of human umbilical vein endothelial cells and acceleration of their apoptosis.8,9 BPI was also identified as a putative binding partner of glypican-4, a surface protein of retinal pigment epithelial cells.10

Murine BPI shows 53% identity and 71% similarity at the amino acid level with human BPI. In the male mouse reproductive system, the BPI gene was reported to be selectively expressed in the testis and epididymis but not in the prostate, seminal vesicle and coagulation glands.11 In the present study, we discovered that mouse BPI was secreted by the epididymal epithelium and was attached to the surface of the spermatozoa plasma membrane. At the same time, BPI was found in the matrix of intact acrosomes, implying a testicular origin. Our results indicated that BPI might have multiple functions in male reproduction.

MATERIALS AND METHODS

Animals
Male C57BL/6 mice and New Zealand white rabbits were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China). All the animal experiments were conducted following the Guide for Care and Use of Laboratory Animals (the ‘NIH Guide’). The protocols for the animal use were approved by the Department of Laboratory Animal Sciences, Shanghai Jiao Tong University School of Medicine (SYXK (Hu) 2008 0050).

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)
Testicular and epididymal total RNA from male C57BL/6 mice was extracted using the TRizol reagent (Invitrogen, NY, USA). Reverse transcription was performed according to the manual of the TaKaRa AMV reverse transcription-PCR kit (TaKaRa, Dalian, China). PCR was conducted on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). The primers used in this study are listed in Table 1. The cycling conditions were as follows: 94 °C for 2 min (one cycle); 94 °C for 15 s, 62 /62.5/64.5 °C for 30 s, 68 °C for 2 min (35 cycles); and 68°C for 10 min (one cycle). PCR products were separated on 1.2% agarose gels. The band intensities were quantified.

1Department of Histology and Embryology, Shanghai Jiao Tong University School of Medicine, Shanghai; *Shanghai Key Laboratory of Reproductive Medicine, Shanghai, China.
*These authors contributed equally to this work.
Correspondence: Dr. C Xu (chenx@shsmu.edu.cn)
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using Image-Pro Plus 5.02 software (Media Cybernetics, Bethesda, MD, USA) and are presented in Supplementary Information.

Prokaryotic expression and purification of recombinant BPI

A 6 x histidine-BPI fusion protein was synthesized using a bacterial expression system. Briefly, the BPI N-terminus was amplified from the cDNA from a 60-day-old mouse testis. The PCR product was subcloned into the pET28a (+) vector. The fusion protein was expressed in E. coli BL21 (DE3) and purified on a His-binding Ni²⁺ chelating affinity resin column using a protocol of the manufacturer’s procedures (Pierce, Rockford, IL, USA).

Polyclonal antibody production and characterization

Male New Zealand white rabbits were immunized with purified recombinant mouse (rm) BPI-N, according to the procedure described in a reference. As described previously, the titer of the antiserum was measured using an enzyme-linked immunosorbent assay. The antiserum was purified with protein A affinity chromatography according to the manual (Millipore, Billerica, MA, USA). The specificity of the antiserum for mBPI was tested by Western blot against the recombinant proteins.

Indirect immunofluorescence

The testes and epididymides were frozen by liquid nitrogen and then sliced into frozen sections of 10 µm thickness. The sections were fixed with methanol for 10 min and then washed by phosphate buffered saline (PBS) three times for 5 min each. The sections were blocked for 1 h with 10% normal goat serum at room temperature. After the PBS washes, purified anti-BPI-N serum (1:100) was applied overnight at 4°C. Adjacent sections were incubated with the preimmune rabbit serum as negative controls. After the PBS washes, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:200) for 1 h in the dark at room temperature, followed by counterstaining with propidium iodide for 10 min. Finally, the sections were washed and mounted in 50% glycerol/PBS. The results were examined under an LSM-510 confocal microscope (Carl Zeiss, Jena, Germany).

Spermatozoa preparation and induced acrosome reaction

Mouse cauda epididymes were dissected and minced in (M16 + 3% bovine serum albumin) media and then incubated at 37°C for 10 min to allow the sperm to swim out. The sperm suspension was adjusted to a concentration of 2 × 10⁷ motile sperm per ml and incubated for 1 h at 37°C, allowing them to capacitate. Subsequently, the sperm suspension was incubated with anti-mBPI-N serum (1:50) for 1 h at 37°C; otherwise, the preimmune rabbit serum was used as a negative control. After PBS washing and fixation in 95% ethanol for 30 min, the sperm were smears on poly-L-lysine-coated slides, air-dried and then incubated with rhodamine (TRITC)-conjugated goat anti-rabbit IgG (1:200) and 0.1 mg ml⁻¹ of FITC-peanut agglutinin lectin (FITC-PNA) for 1 h in the dark at room temperature. The slides were washed and mounted in 50% glycerol/PBS. Digital photographs were taken using an LSM-510 confocal microscope and analyzed by the associated software.

Otherwise, the acrosome reaction was triggered by incubation with 10 μmol l⁻¹ of the calcium ionophore A23187 (Sigma–Aldrich, St. Louis, MO, USA) for 1 h at 37°C, before following the indirect immunofluorescence procedure described above.

Immunoelectron microscopy, post-embedding labeling

Mouse cauda spermatozoa prepared by the swim-out method were fixed in 4% paraformaldehyde for 30 min at room temperature. The spermatozoa were dehydrated in graded ethanol and centrifuged at 500 g. The pellet was then embedded overnight in LR white resin (medium grade; Electron Microscopy Sciences, Fort Washington, PA) at 56°C. Ultrathin sections were prepared and mounted onto nickel grids. The sections were incubated with anti-mBPI-N serum (1:25) diluted in (PBS-T + 5% goat serum); otherwise, control sections were incubated with preimmune rabbit serum, followed by incubation with 10 nm gold-conjugated goat anti-rabbit IgG (GE Healthcare Bioscience, Carlsbad, CA, USA). Between each step, the grids were washed three times with PBS-T for 10 min at room temperature. After staining with uranyl acetate and lead citrate, the ultrathin sections were examined by H-600 electron microscope (Hitachi, Tokyo, Japan).

RESULTS

As shown in Figure 1, the mouse BPI gene became detectable as early as 1 day post-partum during postnatal development. The expression level of BPI in the testis increased quickly within the first 22 days, by which round spermatids appeared in the seminiferous tubules. Afterwards, the BPI expression remained at a stable level, consistent with the dynamics of the first spermatogenic wave (Figure 1). This finding implied that the BPI expression might respond to androgens. Notably, the increase in the BPI expression in epididymis started at 6 day post-partum, which was earlier than that in the testis, suggesting that the BPI expression in these two organs was regulated independently.

Next, to evaluate the protein expression level of BPI, we prepared anti-BPI rabbit polyclonal serum that exhibited high specificity and sensitivity (Figure 2 and Supplementary Figure 1). Unfortunately, we could not obtain a reliable BPI signal in the mouse testis (data not shown). However, the BPI expression exhibited a region-specific pattern throughout the epididymis. The epididymal epithelium consists of several cell types, for example, principal, basal, clear, narrow, halo and apical cells, with individual functions. Principal, basal and halo cells

Table 1: Primer sequences used in this study

| Templates (mouse mRNA) | Primers (5’–3’) | Annealing temperature | Product length |
|------------------------|-----------------|-----------------------|----------------|
| BPI–full length        | F: TAAATTCCTAGCATGACTGGGCTGCG | 62.5°C | 1483 bp |
|                        | R: GTCGCTGCCTCTTCTGTGGCCAAT  | (55–56°C) | 1515 bp |
| β–actin                | F: AGGTGAACAGCTTCTTGCTG  | 62°C | 188 bp |
|                        | R: GCTGCTGCTCAACACCTCAAC |  |  |
| BPI–N                  | F: ATGATCTCCCAGAAGGGTCTGGACTTCG  | 64.5°C | 700 bp |
|                        | R: TTAATGGGCTGTTGAATTCGCTTC | (157–831 bp) |  |

BPI: bactericidal/permeability-increasing protein

Figure 1: RT-PCR analysis of BPI mRNA expression in postnatal mouse testis and epididymis. Lanes 1d, 3d, 6d, 14d, 22d, 30d and 60d represent the results from mice that were 1, 3, 6, 14, 22, 30 and 60 days post-partum, respectively. BPI: bactericidal/permeability-increasing protein; RT-PCR: reverse transcription-polymerase chain reaction.
appear throughout the epididymis. Clear cells are widespread except in the initial segment. In contrast, apical cells and narrow cells only have been found in the initial segment and the caput section. As shown in Figure 3, we found the highest BPI signals in the initial segment. Within the caput section, the BPI signals were spread out in epithelial cells while concentrated at the apical cytoplasm (Supplementary Figure 2). Moreover, the signals in the narrow cell cytoplasm were much higher than those in principal or other cell types (Supplementary Figure 2). The BPI expression then gradually decreased in the corpus and cauda parts and was restricted to the clear cells (white arrowhead).

In addition, BPI-positive signals were detected in the epididymal lumen along with the seminal fluid. These findings led us to explore the localization of the BPI that is associated with spermatozoa. Our results revealed that BPI could attach to the acrosome zone of the spermatozoa plasma membrane before the acrosome reaction (Figure 4, white arrow). Surprisingly, after the induced acrosome reaction, the BPI-positive signals dispersed across the entire sperm head; this dispersal was accompanied by the disappearance of the acrosome structure labeled by PNA-FITC (Figure 4, blue arrow). Furthermore, BPI showed a tendency for enrichment in the equatorial segment (Figure 4, yellow arrow), the initiation site for sperm-egg fusion during the fertilization.

These findings raise the question of the origin of BPI. Was the BPI intrinsically expressed in the acrosome and then exposed later or was the BPI merely secreted by the epididymal epithelia and redistributed after the acrosome reaction? To address this question, we performed an immunoelectron microscopy assay. Our results showed that the BPI signal localized within intact sperm along the acrosome or in the residual cytoplasm (62 ± 18 spots per acrosome, n = 3), while no signal resulted from the preimmune serum controls (Figure 5). This result implied a testicular origin of the BPI protein, which was stored in the sperm head until released during the acrosome reaction. In conclusion, there could be both testicular and epididymal origins of the BPI proteins that are associated with spermatozoa.

**DISCUSSION**

Spermatozoa are continuously generated from spermatogenesis and then transported to the epididymis for functional maturation. The epididymis is a male accessory sex organ consisting of the caput (head), corpus (body) and cauda (tail) segments. It is responsible for sperm surface remodeling, sperm storage and protection. Specifically, principal cells comprise the predominant population with a significant secretory function. Narrow cells are involved in endocytosis and also secrete H+ ions into the lumen by recycling H+ across the apical plasma membrane. Clear cells are large active endocytic cells that normally take up the contents of the cytoplasmic droplets released by spermatozoa. In general, the continual secretion and endocytosis processes throughout the epididymis epithelia are precisely regulated in a spatial manner. Consequently, the composition of luminal fluid changes progressively along the epididymal duct, contributing to the microenvironment that is essential for sperm maturation.

Epididymal secretion products include antimicrobial proteins or peptides, such as mucins and defensins, which could play a protective role at the genital epithelium/bacteria interface. However, these antimicrobial proteins might also be involved in sperm maturation and fertilization. As an example, Bin1, a member of the β−defensin family that expressed in the caput epididymis, is bound to sperm heads and promotes progressive motility by inducing mitochondrial calcium uptake. In contrast, Crisp-1 is expressed mainly in the corpus and cauda epididymis and likely participates in capacitation as well as sperm–egg interactions.

BPI is a cationic antimicrobial protein and plays an important role in the innate immunity against gram-negative bacteria. Although originally identified as a constituent of neutrophil azurophilic granules, BPI expression was found in the human mucosal epithelium, including the female genital tract. Therefore, we speculated that BPI could be expressed along the epithelia of male reproductive tracts as part of the defense against microbial invasion. Unexpectedly, the BPI expression was limited in the mouse testis and epididymis, but not in the lower part of male mouse reproductive tract. This finding indicated that the function of BPI in the male reproductive system might extend beyond the conserved antibiotic effect. In this study, we revealed a unique expression pattern of the BPI protein in the epididymal epithelia; the BPI protein was mainly located in the initial segment and the caput section, but was apparently downregulated in the corpus and cauda portions. In detail, the BPI expression was higher in narrow and clear cells (Figure 3 and Supplementary Information). Given the endocytotic functions of these cell types, this expression appeared to reflect the active metabolism and recycling of BPI protein in the epididymis. Therefore, we supposed that the epididymis-secreted BPI attached to the acrosomal...
region of the sperm head and participated in the modification of the plasma membrane that contributes to the maturation of spermatozoa.

Furthermore, we found BPI in intact sperm, which demonstrated its testicular origin (Figure 5). Thus, BPI became exposed and then diffused after the acrosome reaction (Figure 4), localizing at the equatorial segment, in particular (yellow arrow). Recent studies demonstrated that most mouse spermatozoa begin the acrosome reaction before contacting the zona pellucida during in vitro fertilization. Therefore, the released BPI might play a part in later stages.

Taken together, the findings reported here indicated a dual origin of the BPI that is associated with mouse spermatozoa. This expression pattern of BPI is similar to that of the antimicrobial protein hCAP18/SOB3, which localizes both in the epididymal epithelia and within human spermatozoa acrosomes and displays zona pellucida-binding activity. Our findings suggested that BPI might perform multiple functions in the male reproductive system, including a protective role against potential microbial infection as well as participation in the sperm maturation and sperm-egg fusion processes. These hypotheses require further investigation.
et al. performed all the experiments unless otherwise indicated. XXY performed the IHC detections, analysed the results and drafted the manuscript. QSG participated in the immunofluorescence assay. CX conceived the study. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

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Supplementary information is linked to the online version of the paper on the Asian Journal of Andrology website.

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