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ORIGINAL ARTICLE

The long pentraxin 3 is a soluble and cell-associated component of the human semen

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Summary

The long pentraxin 3 (PTX3) is a multifunctional soluble pattern recognition receptor, involved in several processes ranging from innate resistance and inflammation to clearance of apoptotic cells and organization of hyaluronic acid-rich extracellular matrices. PTX3 is also a novel marker in several pathological conditions of infectious, inflammatory, or autoimmune origin. This study was designed to assess whether PTX3 is expressed in the male reproductive tract and whether PTX3 interacts with human spermatozoa influencing their function. Here we show for the first time by immunohistochemistry that PTX3 is expressed in the male genital tract in perivascular connective tissue, in endothelial cells, in the interstitium, and in the cytoplasm of prostatic epithelial glandular cells; PTX3 was detectable in seminal plasma in variable levels, which correlated with the percentage of normal spermatozoa. Moreover, PTX3 binds to spermatozoa, in particular with immotile cells, localizing in the neck and in the subacrosomial region. Finally, recombinant PTX3 did not interfere with sperm motility.

Introduction

The long pentraxin 3 (PTX3) is a member of the pentraxin superfamily, a family of proteins characterized by a pentameric structure that are highly conserved during evolution (Garlanda *et al.*, 2005; Bottazzi *et al.*, 2006). PTX3 plays an important role in resistance against selected pathogens. It is produced by innate-immunity cells in response to pro-inflammatory signals and acts as a predecessor of antibodies, regulates complement activation, facilitates pathogen recognition by phagocytes and directly recognizes some selected fungal and bacterial microorganisms such as *Aspergillus fumigatus*, *Pseudomonas aeruginosa* and *Salmonella typhymurium* (Garlanda *et al.*, 2005; Bottazzi *et al.*, 2006). PTX3 also binds apoptotic cells (Rovere *et al.*, 2000) and may contribute to editing recognition of apoptotic cells vs. infectious non-self (Baruah *et al.*, 2006). In addition, there is evidence for a regulatory role of PTX3 in non-infectious inflammatory reactions (Souza *et al.*, 2002; Salio *et al.*, unpublished data). In humans, increased levels of PTX3 are found in different infectious diseases (Muller *et al.*, 2001; Mairuhu *et al.*, 2005), in some autoimmune disorders (Luchetti *et al.*, 2000; Fazzini *et al.*, 2001) and in inflammatory conditions, reflecting in particular the involvement of the vascular bed (Latini *et al.*, 2004).

Although PTX3 was initially identified as a molecular component of the immunological network, more recent evidence has demonstrated that it also plays a crucial and non-redundant role in female fertility. Data in mice revealed that PTX3 deficiency is associated to female infertility and that this defect is a result of an abnormal cumulus oophorus characterized by an unstable extracellular matrix in which cumulus cells are dispersed instead of radiating out from a central oocyte (Varani *et al.*, 2002; Salustri *et al.*, 2004). More specifically, PTX3 is produced by cumulus cells and localizes in the hyaluronic acid (HA)-rich extracellular matrix of the cumulus oophorus where it plays a pivotal role in the formation and stabilization of this matrix (Salustri *et al.*, 2004).

Follicular development, oocyte competence, luteinization, implantation, embryo development and delivery are conversely not impaired. Data in human are less abundant but tend to confirm these findings. Cumulus cells express PTX3 and PTX3 protein is present in the cumulus matrix and follicular fluid (Zhang *et al.*, 2005; Paffoni *et al.*, 2006). Real time polymerase chain reaction (RT-PCR) data also show higher levels of PTX3 mRNA in cumulus cells from oocytes, which develop in good quality embryos, indicating in PTX3 a possible marker for oocyte quality (Zhang *et al.*, 2005), although this is not the case for PTX3 protein present in the follicular fluid (Paffoni *et al.*, 2006).

Having found that PTX3 is an essential component of the extracellular matrix of the cumulus oophorus, it was hypothesized that the interaction between the spermatozoa and PTX3 may have a role in in vivo fertilization (Salustri *et al.*, 2004). In line with this hypothesis, Salustri *et al.* documented that murine spermatozoa bound soluble PTX3 and that the binding site was localized in the subacrosomial region (Salustri *et al.*, 2004). The role of the molecule would be merely confined to the interaction between the spermatozoa and the egg as PTX3-deficient male mice were shown to be normally fertile (Varani *et al.*, 2002; Salustri *et al.*, 2004). Interestingly, yet another pentraxin is involved in fertility: apexin, a long pentraxin of guinea pig, is localized in testis, and in particular in sperm, where it is involved in the acrosomial reaction (Noland *et al.*, 1994).

Despite experimental evidence in animals suggesting a role of PTX3 also in spermatozoa function, no data are available in humans. In order to gain insights into the involvement of PTX3 in male fertility, we have designed a study aimed to assess whether PTX3 is expressed in the male reproductive tract and whether PTX3 interacts with human spermatozoa influencing their function.

Materials and methods

Subject

Between April 2005 and January 2006, 86 unselected patients referring to the Infertility Unit, Fondazione Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena were offered to enter the study. They were recruited at the time of referral for semen evaluation and were asked to donate the part of their semen, which was not used for the analysis. A questionnaire regarding the general health condition was filled out by each patient at the time of semen collection in order to exclude patients with malignancies and/or systemic inflammatory diseases. Thirteen further healthy fertile donors accepted to donate a semen sample exclusively for research purposes.

For immunohistochemistry studies on male genital tract, for each type of tissue we used three organs, obtained from three different patients. Their mean age was 61 years (range 26–85). This was a retrospective study performed on tissues obtained from surgery without inclusion or exclusion criteria. Tissues, which were morphologically normal and not involved in any pathological conditions were selected for immunohistochemistry as described below:

Seminal vesicles were obtained from patients with prostatic carcinoma not involving extraprostatic tissues. Seminal vesicles were not involved in neoplasia; prostatic tissues were obtained from patients with prostatic hyperplasia that was limited to the central area of one lobe. For immunohistochemistry, we selected the glandular and stromal components of the uninvolved part of the gland, mainly the contralateral lobe, testis; testicular Leydig cells and epididymis were obtained from patients with small tumours, not exceeding 0.2–0.3 cm in diameter. The components used for immunohistochemical analysis were located far from the neoplasia and were morphologically normal.

All subjects gave a written consent prior to entry into the study. Approval for the study was granted by the local Institutional Review Board.

Semen collection

Semen specimens were collected by masturbation after 3–5 days of ejaculatory abstinence. In patients referring for semen analysis, the sample was analysed according to World Health Organization (WHO) (1999) guidelines using Kruger/Tygerberg Strict Criteria form sperm morphology assessment. Part of the semen, which was not used for the analysis, was centrifuged at 350 ***g*** for 10 min at room temperature to separate them from spermatozoa. Samples were transferred into cryovials and stored at −20 °C until use. In fertile donors, semen was treated by standardized swim-up procedure. Briefly, ejaculates were diluted to 10 mL using Sperm Washing Medium (Irvine Scientific, Santa Ana, CA, USA), centrifuged at 350 ***g*** for 10 min and the supernatant discarded; the pellet was resuspended in 5 mL of the same medium and centrifuged at 350 ***g*** for further 10 min. The supernatant was removed and an aliquot of 1 mL of Sperm Washing Medium was overlaid over the pellet and incubated at 37 °C for at least 30 min. After this period, the upper 0.5 mL was pipetted into a clean tube and the sperm concentration and motility were evaluated. This fraction contained >99% progressive motile spermatozoa while the remaining isolated pellet contained almost exclusively immotile or non-progressive motile spermatozoa. Functional evaluation and immunofluorescence analysis were immediately performed.

Immunohistochemistry

Pattern and site of expression of PTX3 were studied by immunohistochemistry on male genital tract tissues: testis, testicular Leydig cells and epididymis (from patients with testicular neoplasia – seminoma and embryonic carcinoma), seminal vesicle (from patients with prostatic acinar carcinoma) and prostate (from patients with prostatic hyperplasia).

Samples were fixed in neutral buffered formalin and embedded in paraffin; haematoxylin-eosin stained sections were examined for histological evaluation. For immunohistochemical analysis, 3 μm paraffin-embedded sections were cut and mounted on Super-frost slides (Bio-optica, Milan, Italy); after dewaxing in xylene and rehydrating in ethanol, the sections were pre-treated in a microwave oven (two cycles for 3 min each at 800 W, in 0.25 mM ethylenediaminetetraacetic acid (EDTA)) and incubated for 2 h with PTX3 affinity-purified rabbit IgG against human PTX3 (250 ng/mL) (Muller *et al.*, 2001). The reactions were revealed by non-biotin peroxidase detection system (BioGenex, San Ramon, CA, USA) with 3,3′ diaminobenzidine freebase as chromogen. Negative controls were obtained by omission of the primary antibody.

ELISA for PTX3

Seminal plasma sample collection, handling and storage were carefully standardized. The Sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) for PTX3 was performed as previously described (Muller *et al.*, 2001). Briefly, ELISA plates (96 well; Nunc Immuno Plate, MaxiSorp; Nunc A/S, Roskilde, Denmark) were coated with 100 ng/well of rat monoclonal anti-PTX3 antibody (mAb) MNB4 diluted in coating buffer (15 mM carbonate, Na_2CO_3 + $NaHCO_3$, buffer pH 9.6) and were incubated overnight at 4 °C. The plates were washed with washing buffer (Dulbecco's phosphate buffered containing 0.05% Tween20) and 300 μL of 5% dry milk were added to block non-specific binding sites. Fifty μL of recombinant human PTX3 standards (100 pg/mL to 2 ng/mL) and unknown samples were added in duplicate, and incubated for 2 h at 37 °C. After three washes with the washing buffer, 25 ng/well of biotin-conjugated PTX3 affinity-purified rabbit IgG were added for 1 h at 37 °C. Wells were extensively washed and incubated with 100 μL of Streptavidin-peroxidase conjugated to dextran backbone (AmDex, Copenhagen, Denmark) diluted 1 : 4000 for 1 h at room temperature. After incubation the plates were washed four times and 100 μL of TMB chromogen (BD Pharmingen, San Diego, CA, USA) were added. Absorbance values were read at 405 nm in an automatic ELISA reader. PTX3 levels in seminal plasma were analysed after addition of EDTA 0.35% final concentration to sample dilution buffer in order to eliminate interference in the assay by calcium-dependent factors. The absence of interfering factors in the assay and its specificity were ruled out by adding known amounts of recombinant PTX3 or mAb MNB4 to the samples.

Fluorescence-Activated Cell Sorting (FACS) analysis and confocal analysis

Sperm for binding assay was obtained from three fertile healthy men as described above. Binding of PTX3 to spermatozoa was performed as previously described for fungi (Garlanda *et al.*, 2002 Nature). In PTX3 binding assay, spermatozoa were constantly maintained in buffers rich in non-specific proteins (e.g. albumin). Cells were isolated through swim-up procedure in sperm washing buffer containing 5% human albumin, blocked with 5% normal goat serum and bovine serum albumin (BSA) in phosphate buffer saline (PBS). Sperm-associated PTX3 and binding of recombinant PTX3 were characterized by both FACS analysis and confocal microscopy using direct biotin-conjugated PTX3 and a PTX3 affinity-purified rabbit polyclonal Ab. A purified rabbit serum was used as irrelevant control. PTX3 binding on spermatozoa was dose-related in the range of 1–50 μg/mL (data not shown).

After the swim-up selection, cells were separated and resuspended in 0.2% BSA in PBS (washing buffer and reagent diluents). To evaluate either the exogenous or endogenous PTX3 binding, 10^6 motile or non-motile cells isolated from the two fractions of the swim up procedure, were incubated with 10 μg/mL recombinant PTX3 (Bottazzi *et al.*, 1997) or left in reagent diluents, respectively for 30 min at room temperature. Cells were then fixed with 1% paraformaldehyde (PFA; Sigma, Milan, Italy) and incubated for 1 h at room temperature with either PTX3 affinity-purified rabbit polyclonal Ab (5 μg/mL), a purified rabbit serum (5 μg/mL), or no Ab, followed by Alexa Fluor 488 conjugated goat anti-rabbit (Invitrogen-Molecular Probes, Invitrogen, San Giuliano Milanese, Italy) as secondary Ab. Fluorescence was analysed using a FACScan cytofluorometer (BD Biosciences, Erembodegem, Belgium) and results are expressed as percentage and mean fluorescence intensity (MFI) values.

The same protocol was used to evaluate PTX3 localization on sperms by confocal microscope analysis. For DNA detection 1 μg/mL Hoechst 33258 (Molecular Probes) was used. After staining, cells were seeded on glass slides and mounted with FluorSave Reagent (Calbiochem, Nottingham, UK) and analysed with an Olympus Fluoview, FV1000, laser scanning confocal microscope. For confocal analysis of the prostate, tissue preparation and incubation with PTX3 affinity-purified rabbit IgG

against human PTX3 were performed as described above for immunohistochemistry. The reactions were revealed by Alexa Fluor 488 conjugated goat anti-rabbit (Invitrogen-Molecular Probes) as secondary Ab. Images (1024 × 1024 pixels) were acquired with a 100 × 1.4 NA Plan-Apochromat oil immersion objective (Olympus, Hamburg, Germany). Differential interference contrast (Nomarski technique) was also used.

Motility experiments

Recombinant human PTX3 was purified from Chinese hamster ovary cells constitutively expressing the protein as described previously (Bottazzi *et al.*, 1997). Recombinant PTX3 contained <0.125 endotoxin units/mL as checked by the Limulus amebocyte lysate assay (BioWhittaker, Inc., Walkersville, MD, USA).

Fresh spermatozoa obtained from 10 healthy fertile donors were used. Immediately after swim-up procedure, for every sperm sample, a 4-well dish was prepared diluting 3×10^6/mL spermatozoa in Sperm Washing medium containing 0, 10, 30 or 100 μg/mL of recombinant PTX3. At fixed times, after 10, 30, 120 min and 24 h, 10 μL of sperm sample with the different concentration of PTX3 were placed on a clean glass slide and covered with a coverslip of 24 mm × 24 mm. After an equilibration period of 1 min, the preparation was examined at room temperature, at a magnification of 400× with a phase-contrast microscope (Eclipse E200, Nikon, Sesto F., Italy) to assess motility. To this aim, at least 200 successive spermatozoa were evaluated. Every evaluation was repeated a second time in order to obtain a mean value of the percentages of motile spermatozoa.

Statistical analysis

The Social Package for Statistical Science (SPSS 12.0 for Windows, Chicago, IL, USA) was used. Data were reported as mean ± SD and/or median (interquartile range, IQR). Wilcoxon test for k repetitive paired data was used to compare motility in the presence of increasing doses of PTX3. Spearman coefficients were employed to evaluate correlations between semen concentration of PTX3 and semen variables. Wilcoxon test for k independent groups was used to evaluate semen concentration of PTX3 according to categorized semen characteristics. $p < 0.05$ was considered statistically significant.

Results

Immunohistochemistry analysis was performed on tissue sections from pathologic male genital tract, with an immunopurified polyclonal antibody anti-PTX3. Tissue distribution of PTX3 in all the tissues examined in the male genital tract (testis, epididymis, seminal vesicle and prostate), suggested that PTX3 is expressed in perivascular connective tissue and, focally, in endothelial cells and in the interstitium (Fig. 1(a–d)). Moreover, in testis, PTX3

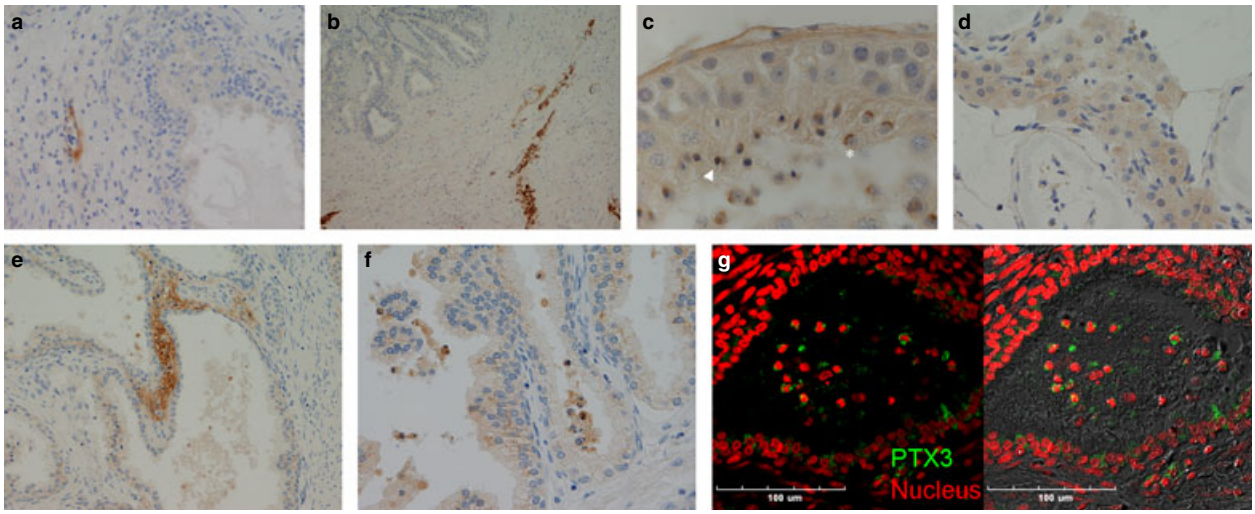

Figure 1 Immunohistochemical analysis of long pentraxin 3 (PTX3) expression in the male genital tract. (a) Epididymis: PTX3 immunoreactivity in endothelial cells. (b) Seminal vesicle: perivascular pattern of distribution (predominantly around lymphatic vessels). Glands were negative. (c) Testis: PTX3 immuno-staining in secondary spermatocytes (asterisk) and spermatidis (arrowhead). (d) Leydig cells: weak PTX3 positivity in the cytoplasm of Leydig cells. (e) Prostate: PTX3 immunoreactivity in prostatic interstitium. 20×. (f) Prostate: focal PTX3 positivity in the cytoplasm of some epithelial glandular cells and in the inflammatory infiltrate localized in glandular lumen. 40×. (g) Confocal analysis of the prostate: PTX3 positivity in the cytoplasm of epithelial glandular cells. Cells were stained for PTX3 (green) and for DNA (red). Differential interference contrast (Nomarski technique) is shown on the right panel.

was found in secondary spermatocytes and spermatids (Fig. 1e); a weak immuno-staining was observed in Leydig cells (Fig. 1f). In prostatic tissues, PTX3 positivity was also found in the cytoplasm of some epithelial glandular cells; areas of prostatic hyperplasia and prostatitis were characterized by a high PTX3 expression in the interstitium and in the inflammatory infiltrate, mainly localized in glandular lumen (Fig. 1c and d). Confocal analysis of the prostate confirmed PTX3 expression in prostatic epithelial glandular cells. Immunoreactivity was also observed in leucocytes and exudates present in the glandular lumen (Fig. 1g). Negative controls were obtained by omission of the primary antibody or in the presence of non-immune purified rabbit serum (not shown).

We then assessed whether PTX3 was detectable in seminal plasma and whether levels correlated with classical variables of semen function. Distribution of PTX3 concentration in 86 semen specimens is illustrated in Fig. 2. The mean ± SD and the median (IQR) were 1.6 ± 4.2 and 0.7 (0.1–1.6) ng/mL, respectively. The molecule was undetectable in 14 cases (16%) while levels were above 2 ng/mL (the upper limit reported in serum from healthy subjects) in 18 specimens (21%) (Muller *et al.*, 2001; Paffoni *et al.*, 2006). PTX3 in seminal plasma from 13 fertile donors was not significantly different from unselected patients, although a tendency for higher levels was recorded [the mean ± SD and the median (IQR) were 2.4 ± 5.3 and 0.8 (0.1–2.2) ng/mL, respectively]. As shown in Fig. 2, levels of the molecule were not normally distributed. The Skewed index (±SE) and the Kurtosis index (±SE) were 6.6 ± 0.3 and 49.9 ± 0.5, respectively. As levels were positively skewed, non-parametric statistics were used for subsequent analyses.

Infertility diagnoses of the 86 recruited patients were grouped into three different categories: male infertility (n = 36), unexplained infertility (n = 36) and others (n = 14) [tubal infertility (n = 6), endometriosis (n = 3) and mixed (n = 5]. The median (IQR) levels of PTX3 in the three groups were 0.6 (0.1–1.5), 0.7 (0.1–2.1) and 1.2 (0.4–1.6) ng/mL, respectively (p = 0.59). Semen characteristics of the specimens are shown in Table 1. The Spearman coefficients of correlation (ρ) between semen concentration of PTX3 and semen volume, spermatozoa concentration, motility and morphology are also reported in Table 1. A statistically significant positive correlation emerged with the percentage of normal spermatozoa (ρ = 0.34, p = 0.004). Moreover, levels of PTX3 according to the commonly used cut-off values for the

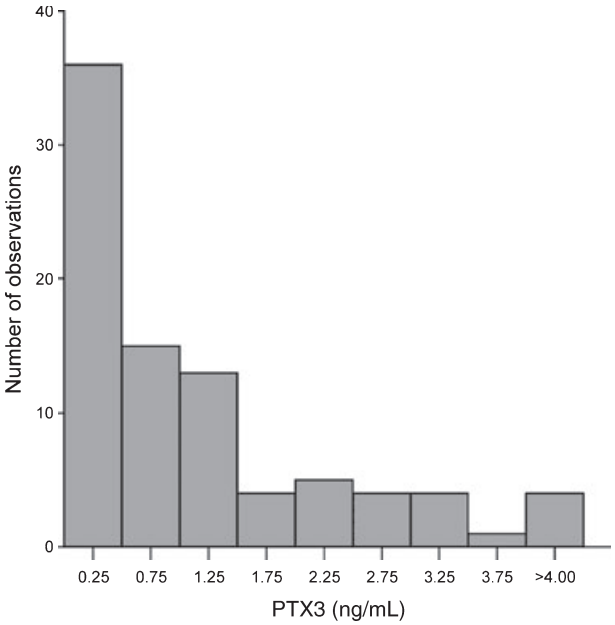

Figure 2 Distribution of semen concentrations of long pentraxin 3 in 86 specimens. The central value per category is reported in the x axis. Data were not normally distributed. A positively skewed distribution was observed. The Skewed index (±SE) and the Kurtosis index (±SE) were 6.6 ± 0.3 and 49.9 ± 0.5, respectively.

Table 1 Sperm parameters and their correlation with long pentraxin 3 (PTX3) levels

	Semen		Correlation with PTX3	
Variable	Mean[a] ± SD[b]	Median[a] (IQR)[c]	Spearman Coeff.	p
Volume (mL)[d]	3.7 ± 1.6	3.4 (2.5–4.5)	−0.19	0.08
Sperm concentration (10^6/mL)[d]	53 ± 39	45 (20–78)	+ 0.13	0.23
Sperm motility[e] (%)	54 ± 15	55 (50–64)	+ 0.17	0.15
Sperm morphology[e] (%)	7.9 ± 5.1	8 (3–11)	+ 0.34	0.004

[a]Values are in ng/mL; [b]SD, Standard Deviation; [c]IQR: Interquartile Range.
[d]Values are referred to 86 patients: in this group mean ± SD and the median (IQR) PTX3 levels were 1.6 ± 4.2 and 0.7 (0.1–1.6) ng/mL.
[e]Patients with azoospermia or cryptozoospermia (<1.10^6 spermatozoa/mL) were excluded (n = 13): in this group mean ± SD and the median (IQR) PTX3 levels were 1.5 ± 4.1 and 0.6 (0.1–1.5) ng/mL.

Table 2 Long pentraxin 3 (PTX3) levels in normal and pathological semen

Variable	*n*	Median[a] (IQR)	*p*
Volume (mL):			
<2	8	1.4 (0.0–3.0)	0.82
2–6	68	0.7 (0.1–1.6)	
>6	10	0.6 (0.1–1.1)	
Sperm concentration (10^6/mL):			
<5	14	0.8 (0.3–2.6)	0.40
5–19	16	0.5 (0.0–1.3)	
>20	56	0.7 (0.1–1.9)	
Sperm motility[b,c] (%):			
<50	18	0.5 (0.0–1.1)	0.09
>50	55	0.7 (0.2–2.0)	
Sperm morphology[b] (%):			
<5	18	0.2 (0.0–1.0)	0.04
5–13	45	0.8 (0.2–2.0)	
>14	10	0.6 (0.3–1.8)	
Round cells (10^6/mL):			
≤1	70	0.7 (0.1–1.6)	0.85
>1	16	0.9 (0.1–1.9)	

[a]Values are ng/mL. IQR: Interquartile range.
[b]Patients with azoospermia or cryptozoospermia (<1 × 10^6 spermatozoa/mL) were excluded (n = 13).
[c]Only one pathological group was set as motility was found to be below 25% only in three patients.

above-mentioned sperm variables were evaluated (1999). Results are shown in Table 2. Again, a statistically significant difference emerged only for the percentage of normal spermatozoa. Specifically, concentration of the molecule appeared to be significantly lower in patients with severe teratospermia. No relationship with the presence of pathological concentration of round cells (>1.10^6/mL) could be demonstrated (Table 2). A subgroup analysis for patients with azoospermia (n = 7) was performed. In this group, the median (IQR) concentration of PTX3 was 0.5 (0.1–1.3) ng/mL. In the remainder of our series, the median (IQR) concentration was 0.7 (0.1–1.6) ng/mL, thus not significantly different (p = 0.77). All previous analyses were repeated considering the total amount of PTX3 per specimen (calculated as concentration × volume of semen) rather than the concentration. Results were essentially similar (data not shown).

To address the potential functional role of PTX3 in male fertility, we studied the interaction between spermatozoa and PTX3. Cytofluorometric and confocal microscopy analysis were performed on spermatozoa (Fig. 3). The results obtained with four donors suggest that PTX3, besides being a soluble component of the seminal plasma, was also associated to cells, in particular to immotile or non-progressive motile cells. In our series, the percentage of PTX3 binding spermatozoa does not correlate with PTX3 levels in the seminal fluid. As shown by FACS analysis, PTX3 was not detected on progressive mobile cells (4.12 ± 2.28%, MFI 49) (Fig. 3a). In contrast, PTX3 was associated to 49.43 ± 5.28% (mean ± SD) immobile spermatozoa, with a MFI of 1.298 (Fig. 3b). Among immobile cells, PTX3 was also associated to 23.4 ± 8.79% 'round cells' (MFI 659) (Fig. 3e).

Cells were also assessed for their ability to bind exogenous recombinant PTX3. As shown in Fig. 3a and b, PTX3 bound more efficiently to immobile or non-progressive mobile than to progressive mobile cells (85.81 ± 8.61%, MFI 17.306 vs. 8.6 ± 2.22%, MFI 418, respectively). 'Round cells' displayed also a strong ability to bind to exogenous PTX3 (65.60 ± 14.83%, MFI 3483) (Fig. 3e).

The single-cell analysis performed by confocal microscopy confirmed these observations and also suggested that the binding site for PTX3 in spermatozoa was predominantly localized in the neck and, to a lesser extent, in the subacrosomial region (a representative experiment is shown in Fig. 3b,d and f).

To exclude the presence of PTX3 stored inside spermatozoa, we performed cytofluorometric and microscopic analyses on permeabilized cells, and ELISA on sperm lysates, but we failed to detect PTX3 (data not shown).

In order to investigate a possible influence of this binding on sperm function, we incubated spermatozoa from 10 different healthy fertile donors with PTX3. We tested the hypothesis that the molecule may have a time- and/or dose-dependent effect on spermatozoa motility. Data on the percentage of motile spermatozoa are shown in Table 3. No significant effect emerged regardless of duration of incubation and the concentration of PTX3 used.

Discussion

The long pentraxin 3 is a multifunctional soluble pattern recognition receptor, involved in several processes ranging from innate resistance to inflammation, from clearance of apoptotic cells to organization of HA-rich extracellular

Figure 3 Binding and localization of long pentraxin 3 (PTX3) in sperm cells. (a, c, e) FACS analysis and (b, d, f) confocal microscopy analysis of endogenous or recombinant (exogenous) PTX3 bound to progressive motile sperms (a, b) (population a in scatter plot), to immotile or non-progressive sperms (c) (population b in scatter plot) and to round cells (e) (population c in scatter plot). Confocal microscopy shows the binding site for PTX3 in spermatozoa predominantly localized in the neck. Cells were stained for PTX3 (green) and for DNA (red). Differential interference contrast (Nomarski technique) is shown on the right of each confocal analysis picture. Cell separation and PTX3 staining were performed as described in Material and Methods.

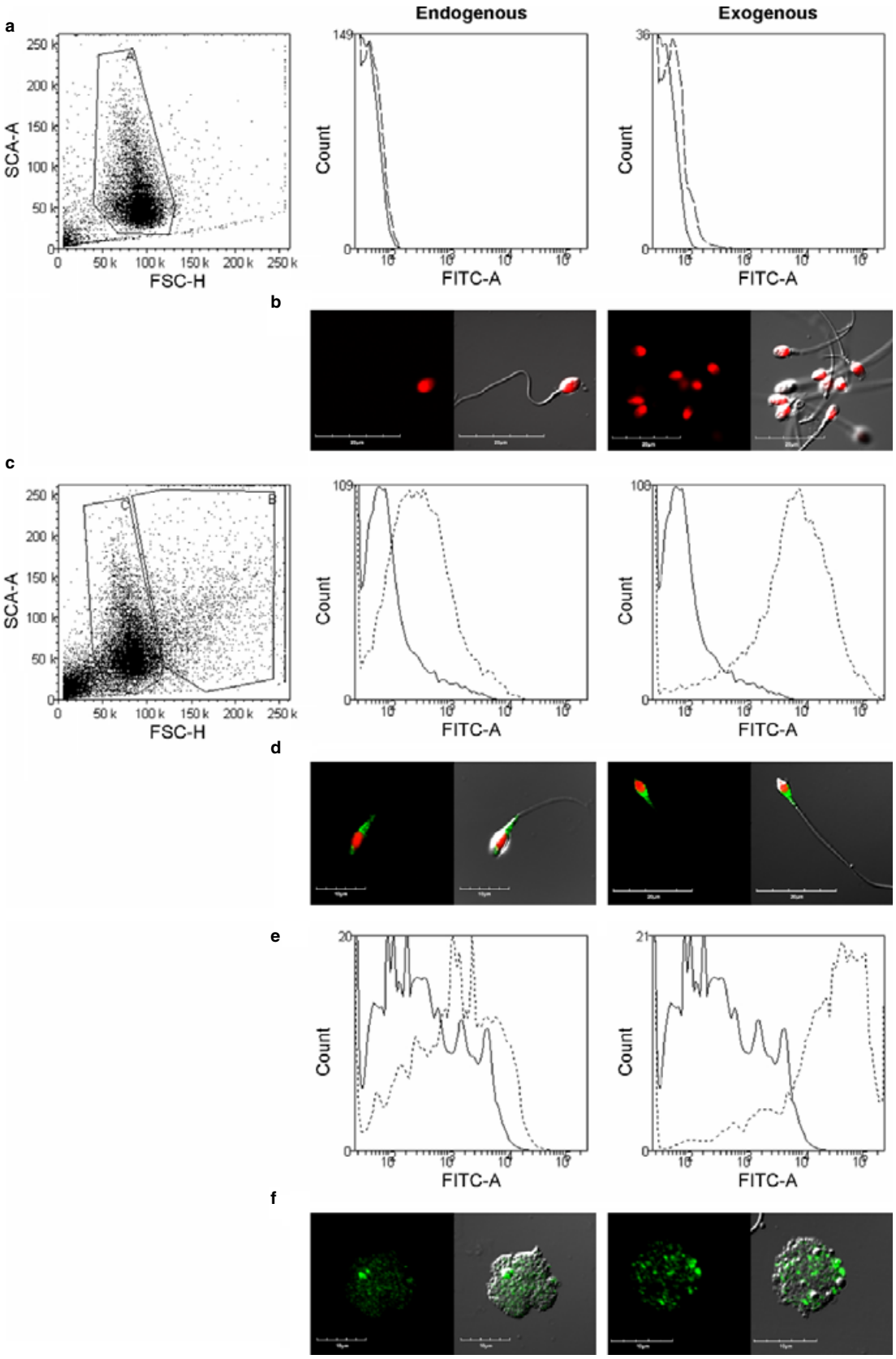

Table 3 Sperm motility in the presence of recombinant long pentraxin 3 (PTX3)

Time point	Control	PTX3			*p*
		10 μg/mL	30 μg/mL	100 μg/mL	
10 min	76 (41–85)	76 (52–85)	78 (45–84)	76 (58–81)	0.87
45 min	65 (33–80)	70 (30–77)	55 (37–76)	54 (35–73)	0.78
120 min	41 (15–54)	35 (15–62)	42 (24–58)	42 (10–70)	0.11
24 h	2 (0–10)	2 (0–11)	2 (0–12)	4 (0–8)	0.97

Median (interquartile range) values from 10 different experiments are reported. Data are expressed as median (interquartile range).

matrices (Garlanda *et al.*, 2005). Capable of being rapidly induced in inflammatory conditions, PTX3 is also a novel marker in several pathological conditions of infectious, inflammatory, or autoimmune origin (Fazzini *et al.*, 2001; Muller *et al.*, 2001; Latini *et al.*, 2004). In this study, we have demonstrated for the first time that PTX3 is expressed in the male genital tract and is a soluble and cell-associated component of seminal plasma. Moreover, we demonstrate that recombinant PTX3 binds human spermatozoa without interfering with sperm motility.

Tissue distribution of PTX3 in male genital tract suggests that PTX3 is expressed in perivascular connective tissue (predominantly around lymphatic vessels) of male accessory glands, and, focally, in endothelial cells and in the interstitium. Immunoreactivity in prostatic glandular cells and exudates suggests that the prostate is an important source of the PTX3 found in semen. Consistently, the prostate carcinoma cell line PC3 expresses PTX3 mRNA and protein (C. Garlanda, unpublished) and cells obtained from benign prostatic hyperplasia secrete PTX3 in basal condition and in particular after pro-inflammatory stimuli (G. Penna, unpublished). Overall, these data suggest that the male genital tract is a source of PTX3 as a physiological component of the seminal plasma. Moreover, PTX3 immunoreactivity was increased in inflammatory conditions and involved inflammatory cells and vessel walls. This pattern of expression is consistent with what has been observed in other tissues (Cetin *et al.*, 2006; M. Nebuloni, unpublished), and suggests that PTX3 might be a diagnostic marker of inflammatory conditions of the male genital tract. It is worth mentioning that in the present study immunohistochemical localization of PTX3 was performed on pathological specimens and confirmative data using normal/healthy tissue specimens are needed.

The presence of PTX3 in male reproductive tract was confirmed in the murine model: RT-PCR was performed on murine testis, prostate, epididymis, ductus deferens, coagulation glands and compared to the heart, that even in normal conditions expresses low levels of PTX3 mRNA. Expression in the testis was low, but in other tissues PTX3 expression was similar to that observed in the heart (data not shown).

In this study, we have evaluated whether PTX3 levels in human semen correlated to classical variables of semen function. To address this point, we recruited 86 consecutive male patients who were referred to our infertility unit to undergo a semen analysis and we evaluated levels of PTX3 in the seminal fluid. The mean and median levels of the molecule were shown to be comparable to levels reported in plasma from healthy patients, for whom concentration is generally between 1.0 ng/mL and 2.0 ng/mL (Muller *et al.*, 2001; Paffoni *et al.*, 2006). Surprisingly, concentration of the molecule was not normally distributed. Levels were indeed markedly positively skewed. In some cases, extremely high seminal levels of PTX3 were documented while in a significant proportion of specimens (16%) the molecule was undetectable. To gain insights into the possible reasons surrounding this observation, we evaluated levels of PTX3 in three different diagnostic groups (male infertility, unexplained infertility and others). This analysis did not show any significant result. We then correlated the seminal levels of PTX3 to classical variables of semen quality. No relationship with semen concentration, semen motility and the presence of round cells was observed. Conversely, a modest positive correlation was documented with sperm morphology ($\rho = 0.34$). Interestingly, immunoreactivity was observed in testis associated in particular to spermatids and spermatocytes, and to a lower extent to Leydig cells. It may be speculated that PTX3 released in seminiferous tubules is a marker of a normal spermatogenesis. It is however unlikely that this release is of biological relevance considering that, in mice the absence of PTX3 is not associated with male infertility. Further studies are required to disentangle this issue.

The frequency of diagnostic groups observed in our series does not reflect proportions usually observed in infertility units (Evers, 2002). The decision to include patients consecutively has indeed lead to the inclusion of a greater proportion of patients with unexplained or male infertility as this kind of patients are generally prescribed semen analysis more frequently.

It is noteworthy that PTX3 is typically expressed in inflammatory conditions and it may be hypothesized that levels of the molecule might be enhanced in inflammatory and/or infective conditions such as prostatitis or other male accessory gland infections. Immunohistochemistry analyses support this possibility as areas of prostatitis were characterized by higher PTX3 expression. In order to clarify possible relationship between PTX3 and diagnostic entities, a future prospective and properly designed study is warranted. In particular, recruited subjects will

have to undergo a complete diagnostic evaluation including a standardized andrological clinical assessment, a testicular and transrectal sonography, a Stamey test and hormonal blood tests. The female partner should also undergo a complete clinical, hormonal and instrumental evaluation.

Conversely, PTX3 concentration was not related to the concentration of seminal round cells whose presence in the seminal fluid is believed to reflect genital inflammatory conditions (Hochreiter, 2003). A future study aimed to specifically address this point is required. Of relevance here is that reliable seminal markers of genital inflammatory conditions are currently lacking (Hochreiter, 2003).

FACS and confocal analysis of endogenous and recombinant PTX3 binding to spermatozoa indicate that PTX3 binds spermatozoa, in particular immobile cells and that the binding site is mainly localized in the neck of the spermatozoa and in part in the subacrosomial region. This last observation is partly in contrast with what was observed in mice, as all murine spermatozoa bound PTX3, and the binding site was shown exclusively localized in the subacrosomial region (Salustri *et al.*, 2004). Interspecies differences have thus to be considered to explain this difference.

The long pentraxin 3 is implicated in the recognition and clearance of apoptotic cells (Rovere *et al.*, 2000; van Rossum *et al.*, 2004). Apoptosis occurs in the testis and in normal spermatogenesis (Muratori *et al.*, 2006). The finding that PTX3 binds with higher affinity immobile sperms, compared to progressive mobile sperms opens the possibility that PTX3 is involved in recognition and modulation of clearance of apoptotic sperms. The linkage between PTX3 and clearance of apoptotic spermatozoa has to be deeply investigated, but in our hypothesis PTX3 could be considered a negative marker of 'sperm health' and PTX3-negative spermatozoa could be used to improve in vitro fertilization results. Sperm motility, vitality and morphology represent primary criteria for in vitro sperm selection. In some cases, sperm samples present reduced or absent sperm motility and, although at the moment it is purely theoretical, PTX3-binding could have an impact in selecting spermatozoa with better fecundity potential among immotile spermatozoa.

The long pentraxin 3 is also involved in regulating complement activation and depending on its presence as soluble or solid phase-associated protein, plays the dual role of inhibitor or activator of the complement cascade (Bottazzi *et al.*, 1997; Nauta *et al.*, 2003). Complement is a major player in innate immunity and is present in the female genital tract. Spermatozoa must therefore evade complement attack in the female genital tract. Complement evasion is achieved by the presence of complement regulators both in seminal plasma and on the spermatozoa (Harris *et al.*, 2006). The presence of PTX3 in normal seminal plasma suggests a potential involvement as complement modulator.

The long pentraxin 3-deficient female mice are infertile as a result of a defect in cumulus matrix assembly. Of relevance here is that the oocytes ovulated by PTX3-deficient mice were not fertilized in vivo but could be fertilized in vitro (Salustri *et al.*, 2004). This observation supports the crucial role of the normal expansion of the cumulus in vivo, a role possibly connected with the presence of a very low number of spermatozoa at the site of in vivo fertilization. In other words, there is the need for sperm to be captured and guided to the oocyte (Eisenbach & Tur-Kaspa, 1999). It has been suggested that the binding of PTX3 to the spermatozoa may be a crucial component of the mechanisms facilitating access of sperm to the oocyte (Salustri *et al.*, 2004). Even though the present study was not useful to clarify this possible role, our experimental data on the influence of PTX3 on sperm motility are consistent with this vision. We failed to observe any effect on motility even at extremely elevated doses of PTX3 and for longer periods of incubation. The interaction of PTX3 with the spermatozoa does not thus influence motility, a crucial function in terms of reproductive competence.

In summary, results from the present study support the following conclusions: 1) The long pentraxin PTX3 is expressed in male reproductive tract, at low level in testis, in epididymis and in seminal vesicles, and at higher levels in prostate in physiological conditions and particularly in hyperplasia and inflammation. 2) PTX3 is a component of the semen, found both in the soluble phase and the cell-associated phase. 3) Semen concentration of PTX3 is highly variable and correlates with the percentage of normal spermatozoa, but causes surrounding this variability are unknown and remain to be clarified. 4) Human spermatozoa, in particular immobile cells, bind PTX3 and the binding site is mainly localized in the neck and in the subacrosomial region of the spermatozoa. 5) Recombinant PTX3 does not interfere with sperm motility.

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