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The 60 kDa heat shock protein in human semen: relationship with antibodies to spermatozoa and *Chlamydia trachomatis*

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The presence of the 60 kDa heat shock protein (hsp60) in seminal fluid and its relationship to sperm autoimmunity or a localized immune response to *Chlamydia trachomatis* were examined. Semen from 64 male partners of infertile couples with no history of a chlamydial infection were investigated. Hsp60 was identified by an enzyme-linked immunosorbent assay (ELISA) using a monoclonal anti-hsp60 antibody bound to wells of a microtitre plate and a polyclonal anti-hsp60 antibody for detection. Antisperm antibodies on motile spermatozoa were detected by immunobead binding, while antichlamydial immunoglobulin (Ig) A and IgG in seminal fluid were identified by a commercial ELISA (SeroELISA; Savyon Diagnostics, Beer-Sheva, Israel). RNA was purified from isolated seminal round mononuclear cells and tested for hsp60-specific mRNA by a reverse transcription polymerase chain reaction ELISA. Hsp60 was present in semen from nine (14.1%) men, 12 (18.8%) men had antisperm autoantibodies, 16 (25.0%) were positive for antichlamydial IgA and 17 (26.6%) had detectable hsp60-specific mRNA. The presence of hsp60 in semen correlated with the occurrence of antichlamydial IgA ($P = 0.0005$), hsp60 mRNA ($P = 0.04$) and antisperm antibodies ($P = 0.05$). Thus, hsp60 was present in a soluble form in semen primarily in men with evidence of immune system activation within their genital tract. The role of hsp60 in promoting or inhibiting immune responses within the genital tract remains to be determined.

Key words: antisperm antibodies/*Chlamydia trachomatis*/hsp60

Introduction

The 60 kDa heat shock protein (hsp60) is produced in mammalian cells in response to various stresses, i.e. elevated temperature, infection, inflammation and toxic chemicals (Young, 1990). Its function appears to be to prevent protein denaturation or inappropriate polypeptide aggregation during the period of stress, thereby allowing the cell to survive and resume normal functions after the stress is alleviated. There is also evidence that this family of proteins may directly inhibit inflammatory responses. The induction of heat shock protein gene transcription is associated with the down-regulation of inflammatory cytokine gene expression (Schmidt and Abdulla, 1988; Snyder *et al.*, 1992).

Our laboratory has been analysing immune mechanisms within the human male genital tract. Asymptomatic male genital tract exposure to *Chlamydia trachomatis*, detected by the presence of antichlamydial immunoglobulin (Ig) A in semen but not in serum from men with no history of a *C.trachomatis* infection, was shown to be associated with elevated levels in semen of T lymphocytes bearing the γδ (γδ T lymphocytes) or the αβ (αβ T lymphocytes) form of the antigen receptor (Munoz and Witkin, 1995). The presence in the ejaculate of spermatozoa with bound autoantibodies (antisperm antibodies) was associated with an increased concentration of γδ T lymphocytes (Munoz *et al.*, 1992). Recently, it has been reported that γδ T cells induce hsp60 expression (Hisaeda *et al.*, 1995). In addition, it has been shown that a large fraction of γδ T lymphocytes are specifically activated by hsp60 (O'Brien *et al.*, 1992). Hsp60 is also a component of pathogenic micro-organisms (Benkirane *et al.*, 1992). Therefore the presence of hsp60 in semen may indicate an infection or inflammatory process. In this study we examined whether hsp60 could be detected in semen from men with antisperm or antichlamydial antibodies, and whether the mononuclear cells in semen were a source of the hsp60.

Materials and methods

Subjects

A total of 64 male partners of infertile couples who sought antisperm antibody testing were studied. The subjects were consecutive, except that men were excluded if they or their partner had ever been diagnosed with a chlamydial infection or pelvic inflammatory disease.

Semen

Semen was obtained by masturbation after 2–5 days of sexual abstinence. Motile spermatozoa were isolated by overlaying the semen samples, after liquefaction, with an equal volume of warm (37°C) phosphate-buffered saline (PBS), incubating at 37°C for 60 min and collecting the upper PBS layer. The remaining semen was centrifuged and mononuclear cells isolated from the pellet fraction by Ficoll–Hypaque density centrifugation as described previously (Munoz and Witkin, 1995). The mononuclear cell fraction contained lymphocytes, monocytes and round immature sperm cells. The final supernatant was stored in aliquots at –80°C until utilized for the determination of antichlamydial IgA and heat shock proteins.

Antisperm and antichlamydial antibodies

IgG and IgA antibodies bound to the surface of motile spermatozoa were visualized by the direct immunobead binding assay (Bronson

et al., 1984). Bead binding to at least 20% of the motile spermatozoa was considered to be significant Cell-free seminal fluid was diluted 1 5 and tested for IgA and IgG antichlamydial antibodies by a commercial enzyme-linked immunosorbent assay kit (SeroELISA, Savyon Diagnostics, Beer-Sheva, Israel) as described previously (Munoz and Witkin, 1995) The IgA assay included a step to remove competing IgG antibodies and rheumatoid factor

Detection of hsp60 in seminal fluid

Hsp60 in cell-free seminal fluid was detected by a sandwich ELISA using a monoclonal antibody to hsp60 (SPA807; StressGen, Vancouver, BC, Canada). The antibody was diluted to 10 μg/ml in 0.1 M carbonate buffer, pH 9.8, and 0 1 ml were added to individual wells of a microtitre plate After an overnight incubation at 4°C, the wells were washed with PBS–0.05% Tween 20; seminal fluids, diluted 1:1 in PBS–Tween, were added to duplicate wells. After 60 min in a 37°C water bath, the wells were washed and 0.1 ml of a 1:500 dilution in PBS–Tween of rabbit antibody to hsp60 (SPA804, StressGen) added. After 60 min at 37°C, the wells were washed and 0 1 ml of a 1.200 dilution of alkaline phosphatase-conjugated goat antibody to rabbit IgG (Kirkegaard and Perry, Gathersberg, MD, USA) added. After a 60 min 37°C incubation, the wells were washed and the alkaline phosphatase substrate, *p*-nitrophenyl phosphate, in 10% diethanolamine buffer (Pierce Biochemical, Rockford, IL, USA) was added. The colour change in the wells was measured at 405 nm using a microtitre plate reader Purified human and *Escherichia coli* hsp60 (StressGen) were utilized as positive controls. A positive result was defined as a value at least three SD above the mean value (an absorbance of 0.351) obtained using 50 randomly selected semen samples.

Measurement of human hsp60-specific mRNA in seminal mononuclear cells

Isolated seminal mononuclear cells were pelleted by centrifugation and 1 μl RNA-Guard ribonuclease inhibitor (Pharmacia, Piscataway, NJ, USA) was added to the pellet. A solution of 0.15 M NaCl, 1.5 mM $MgCl_2$, 0.65% Nonidet P40 in 10 mM Tris–HCl, pH 7 5, was then added to lyse the cells and release the nucleic acid. Following centrifugation, the supernatant was removed and admixed with an equal volume of 7 M urea, 1% sodium dodecyl sulphate, 0.35 M NaCl, 10 mM EDTA in 10 mM Tris buffer. RNA was extracted by addition of an equal volume of a 50 50 1 mixture of phenol:chloroform:isoamyl alcohol. The samples were vortexed, centrifuged and the RNA re-extracted. The RNA was precipitated by adding 100% ice-cold ethanol, 3 M sodium acetate and incubating overnight at –20°C. The precipitate was washed in 70% ethanol, dried by desiccation and resuspended in diethyl pyrocarbonate-treated water Samples were stored at –80°C.

The RNA was treated for 15 min with 1 unit of amplification grade deoxyribonuclease I (DNase, Gibco BRL Life Technologies, Grand Island, NY, USA) to remove any contaminating DNA. The DNase was then inactivated by adding 2 mM EDTA and heating for 10 min at 65°C. The RNA was then reverse transcribed into cDNA utilizing 600 units of murine Maloney leukaemia virus reverse transcriptase (Gibco BRL Life Technologies) in 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 0.5 mM each dATP, dTTP, dCTP and dGTP (Promega, Madison, WI, USA), 1330 units/ml RNasin ribonuclease inhibitor (Promega) and 50 μg/ml oligo dT_{15} primer (Promega) Samples were incubated for 60 min at 37°C, heated at 95°C for 5 min to inactivate the reverse transcriptase and stored at –80°C

Aliquots of cDNA were admixed with reaction buffer [10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dATP, dCTP and dGTP, 190 μM dTTP, 10 μM digoxigenin-11-2[1]-deoxyuridine-5[1]-triphosphate (dig-11-dUTP; Boehringer Mannheim, Indianapolis, IN, USA), 0.15 mM oligonucleotide primer pairs specific for a region of the human hsp60 gene (StressGen) and 1 25 units *Taq* DNA polymerase] in a total volume of 50 ml. Samples were subjected to one cycle at 95°C for 3 min, 48°C for 30 s and 72°C for 1.5 min, followed by 28 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 1.5 min. This was followed by 5 min at 72°C The polymerase chain reaction (PCR) products were then subjected to electrophoresis on 1 7% agarose gels, stained with ethidium bromide and the DNA visualized under ultraviolet light. The hsp60 primers yielded a band of 309 bp. All cDNA samples were also tested for β-actin DNA to ensure that cDNA synthesis occurred in our samples

To quantitate the amplified PCR products, 10 μl aliquots of the dig-labelled products were denatured in alkaline denaturation buffer (Boehringer Mannheim) at 25°C for 10 min and hybridized with 7.5 pmol/ml of a biotinylated hsp60-specific oligonucleotide probe (Oswel DNA Service, Southampton, UK) The dig-labelled biotin PCR product complexes were then incubated in wells of a streptavidin-coated microtitre plate for 3 h at 42.9°C, and the bound product was detected using peroxidase-conjugated anti-dig antibody (Boehringer Mannheim) and the colorimetric substrate ABTS Wells containing no cDNA, and PCR products negative for hsp60 DNA, were used as negative controls. Known concentrations of human DNA were amplified by PCR using the same primer pairs and utilized to derive a standard curve to convert the ELISA readings to ng DNA equivalents/ml. The sensitivity of the assay was <9 ng/ml

Statistics

Fisher's exact test was used to evaluate differences between groups in the numbers of subjects positive for seminal hsp60.

Results

Hsp60 in seminal fluid

Utilizing monoclonal antibody SPA807, hsp60 was detected in nine (14%) of the semen samples tested (Table I). The positive values ranged from 4.5 to 9.0 SD above the control mean. The individual values for the samples positive for hsp60 are shown in Table I.

The sperm characteristics (count, morphology and percentage progressive motility) of the patients whose semen was positive for hsp60 were within the normal range and did not differ from those of the other patients (data not shown).

Antibodies to C.trachomatis in seminal fluid

Only one subject had IgG antibodies to *C.trachomatis* detected in his seminal fluid. In contrast, 16 samples (25.0%) were positive for antichlamydial IgA in seminal fluid. This agreed with our previous findings of a 29.2 (Munoz and Witkin, 1995) and 24.7% (Witkin *et al.*, 1995) prevalence of these antibodies in semen from similar populations. The relationship between antichlamydial IgA and hsp60 in seminal fluids is shown in Table II. Antichlamydial IgA was detected in seven (77 7%) of nine samples with hsp60 and in only nine (16.4%) of 55 samples that lacked hsp60. The combined presence of the two molecules was highly correlated ($P = 0.0005$).

Antisperm antibodies on motile spermatozoa

Antisperm autoantibodies were detected on the surface of motile spermatozoa from 12 (18.8%) subjects. IgA antibodies

Table I. The presence of immunoglobulin (Ig) A antibodies to *Chlamydia trachomatis* (Ct), mRNA to heat shock protein 60 (hsp60) and antisperm antibodies in semen of men positive for seminal hsp60

Subjects with hsp60 in semen	Absorbance (405 nm)		Hsp60 mRNA (ng/ml)	Antisperm antibodies
	Hsp60	Anti-Ct IgA		
1	0 611	0.247	<9.0	None
2	0 515	0.696	<9.0	IgA
3	0 501	0 552	111	IgG
4	0 494	0 327	122	None
5	0 480	0 524	88	IgA
6	0.468	0.086	<9 0	IgA
7	0.434	0 107	<9 0	None
8	0.432	0.278	93	None
9	0.431	0.455	20	None

Table II. Relationship between heat shock protein 60 (hsp60) in seminal fluid and anti-*Chlamydia trachomatis* (Ct) immunoglobulin (Ig) A in semen, hsp60 mRNA in seminal mononuclear cells and antisperm antibodies on ejaculated spermatozoa

Hsp60 in seminal fluid	No of subjects	No. (%) positive		
		Anti-Ct IgA	Hsp60 mRNA	Antisperm antibodies
Present	9	7 (77 7)[a]	5 (55 5)[b]	4 (44 4)[c]
Absent	55	9 (16 4)	12 (21 8)	8 (14.5)

[a]P = 0 0005; [b]P = 0 04; [c]P = 0 05

were present in seven subjects, bound to 30–100% of motile spermatozoa, while six men were positive for IgG antisperm antibodies, bound to 30–95% of motile spermatozoa. Individual results for the hsp60-positive semen samples are shown in Table I. The relationship between antisperm antibodies and hsp60 in seminal fluid is shown in Table II. Antisperm antibodies on ejaculated spermatozoa were present in four (44.4%) of nine men with hsp60 in their semen as opposed to eight (14.5%) of 55 men whose semen lacked hsp60 (P = 0.05). Three men with only IgA antisperm antibodies and one with only IgG antisperm antibodies were positive for hsp60.

Hsp60 mRNA in semen mononuclear cells

mRNA specific for human hsp60 was detected in mononuclear cells isolated from 17 (26.6%) semen samples. The positive samples ranged from 20 to 122 ng/ml. The relationship between hsp60 mRNA in mononuclear cells and hsp60 in seminal fluid is shown in Tables I and II. Hsp60 mRNA was present in mononuclear cells from five (55.5%) of nine men whose semen contained hsp60 as opposed to 12 (21.8%) of 55 men whose semen lacked hsp60 (P = 0.04).

Discussion

Hsp60 was demonstrated in a soluble form in human seminal fluid, in association with genital tract autoantibody formation (antisperm antibodies) or a genital tract exposure to *C.trachomatis* (antichlamydial IgA). In five of nine cases with seminal hsp60, human hsp60 gene transcription was detected in mononuclear round cells. This suggests that the immature sperm cells, αβ and/or γδ T lymphocytes and/or monocytes/macrophages present in this fraction are a source of hsp60 in semen. Because hsp60 was present in some semen samples without concomitant hsp60 mRNA expression by mononuclear cells, other cells in the male genital tract, or an infectious agent, may be an additional source of seminal hsp60. The presence of hsp60 mRNA in 17 semen samples while only seven were positive for hsp60 indicates that either the reverse transcription PCR is more sensitive than the ELISA or the mRNA detected was not always translated into protein and/or released from cells in the absence of immune activation. Our ELISA assay specifically measured soluble hsp60 and not cell-associated hsp60. The lack of a correlation between hsp60 mRNA and antichlamydial and antisperm antibodies leads us to favour the latter explanation.

Another heat shock protein with a molecular weight of 70 kDa (hsp70) has been shown to be present on human spermatozoa and in seminal fluid (Miller *et al.*, 1992), but thus far there are no reports of sperm-associated hsp60.

We are unaware of published reports of hsp60 being present in other biological fluids. The release of hsp70 from rat embryo cells into the culture medium has been demonstrated (Hightower and Guidon, 1989). It was proposed by these authors that the release of heat shock proteins may serve to protect adjacent cell types, which are incapable of heat shock protein synthesis, from a localized stress.

Hsp60 protein synthesis is an indicator of immune activation. Inflammatory cytokines as well as reactive oxygen species, both products of an activated immune response, induce hsp60 expression (Peetermans *et al.*, 1995). Thus, the association of hsp60 with a humoral immune response to *C.trachomatis* or spermatozoa suggests that immune stimulation is currently being elicited in the genital tracts of these men. This is consistent with our previous studies of increased levels of αβ and γδ T lymphocytes in semen from men with antichlamydial and antisperm antibodies. The data suggest, therefore, that those men with both antichlamydial IgA and hsp60 in their semen may be currently asymptomatically infected with this organism. The existence of a non-productive and chronic chlamydial infection which is, nevertheless, capable of eliciting an immune response has been demonstrated previously in trachoma (Schachter *et al.*, 1988) and in the female genital tract (Patton *et al.*, 1994). However, the presence of anti-chlamydial IgA in the semen of some men who were negative for seminal hsp60 and vice versa indicates that these two markers are not always related. Certainly, hsp60 expression can be elicited by other infectious or non-infectious factors. Similarly, the observation that not all men with antichlamydial or antisperm antibodies were positive for seminal hsp60 suggests that the induction of hsp60 protein synthesis in different individuals is dependent on genetic factors as well as the particular stimulus inducing antibody formation in the male genital tract.

The possible function(s) of hsp60 in human seminal fluid and whether a proportion of the hsp60 and/or hsp60 mRNA we detected in semen originated from γδ T cells remains to be determined. γδ T cells can be activated by infectious agents (Modlin *et al.*, 1989) to release cytokines (Follows *et al.*,

1992), which may then induce hsp60 gene expression in other cells (Ferm *et al*, 1991) Although the sequence of events remains unclear, further exploration of the association between asymptomatic *C.trachomatis* infection of the male genital tract, γδ T cell activation, antisperm autoimmunity and hsp60 expression could open new perspectives to our understanding of the immune response within the human male genital tract

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