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<td>Author(s)</td>
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<td>Citation</td>
<td>Nagasaki University (長崎大学) 博士 (医学) (2019-09-04)</td>
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<td>Issue Date</td>
<td>2019-09-04</td>
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<td>URL</td>
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Comprehensive immune complexome analysis detects disease-specific immune complex antigens in seminal plasma and follicular fluids derived from infertile men and women

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ARTICLE INFO

Keywords:
Immunocomplex antigen
Immunocomplexome analysis
Spermatogenic dysfunction
Endometriosis

ABSTRACT

Background: Autoimmune reactions and subsequent inflammation may underlie spermatogenic dysfunction and endometriosis-related infertility. The aim of this study is to identify disease-specific antigens in immune complexes (ICs) in seminal plasma (SP) and in follicular fluid (FF).

Methods: Immune complexome analysis, in which nano-liquid chromatography-tandem mass spectrometry is employed to comprehensively identify antigens incorporated into ICs in biological fluids, was performed for specimens collected from infertile couples undergoing assisted reproduction. Forty-two male patients consisting of subjects with oligozoospermia (n = 6), asthenozoospermia (n = 8), and normal semen analysis (n = 28). Fifty-eight female patients consisting of subjects with ovarian endometriosis (n = 10) and control women without disease (n = 48).

Results: Four disease-specific antigens were identified in subjects with oligozoospermia, while five disease-specific antigens were detected in subjects with asthenozoospermia, some of which are involved in spermatogenesis. Eight antigens were detected only in subjects with endometriosis.

Conclusion: Functional characteristics of disease-specific antigens were found to correspond to the pathogenesis of male and female infertility. The formation of ICs may contribute to spermatogenic dysfunction and endometriosis-related infertility via loss of function of the related proteins. Immune complexome analysis is expected to be a valuable tool for the investigation of novel diagnostic methods and treatment strategies for infertility.

1. Introduction

Local immunity may play an important role in human reproduction, and disorders in local immunity can be the cause of male and female infertility [1,2]. Spermatogenic dysfunction is a major cause of male infertility, though its pathogenesis is not fully understood. Inflammation in the male reproductive tract may disrupt spermatogenesis and sperm function. Destruction of testicular microstructures can induce immunity against sperm [3]. Although autoimmune orchitis and epididymitis may be rare occurrences, these conditions may relate to immunological male infertility [4]. However, even subclinical inflammation and local autoimmune reaction in the male reproductive tract can be a cause of male infertility. To permit normal spermatogenesis and fertility, the...
mammalian testis is maintained as an immune-privileged organ wherein immunogenic germ cells are protected from immune surveil-

lance [1]. The Sertoli cell barrier, also known as the blood–testis barrier (BTB), plays an important role in the construction of this unique mi-

croenvironment [5]. When the BTB is impaired due to infection, injury, or obstruction of genital ducts, a large amount of sperm antigen is ex-

posed to immune cells by leakage or infiltration, leading to sperm im-

munity [6]. Inflammation of the testis may affect male reproductive function. Subacute and chronic inflammation of the testis and epidi-

dymus are asymptomatic in the most patients, and there are no reliable
clinical diagnostic measures [3]. As a local body fluid of the male re-

productive tract, seminal plasma (SP), mediates male fertility by sup-

porting sperm metabolism, modulating sperm function, and protecting sperm against the damage induced by the immune system via sup-

pression of immune activity [7,8]. SP, which is formed by secretion from male reproductive organs including the epididymis, seminal ve-

sicles, prostate gland, and Cowper's gland, contains many kinds of

plasma constituent antigens in ICs, we

As mentioned above, autoimmune reactions and subsequent in-

flammation may underlie spermatogenic dysfunction and en-

dometriosis-related infertility. However, while SP and FF are local body fluids of the gonads and reproductive tract, it is unknown what com-

ponent(s) of SP and FF are recognized as auto-antigens, thereby trig-

gering inflammation in the corresponding tissues. Immune complexes

(ICs) are formed by noncovalent interactions between foreign antigens or autoantigens and antibody molecules [25]. Enhanced formation and
defective clearance of ICs occurs in autoimmune diseases [26]. In order
to comprehensively identify and profile constituent antigens in ICs, we
developed a proteomic strategy, designated immune complexome analysis, in which ICs are separated from whole serum and then sub-

jected to direct tryptic digestion and nano-liquid chromatography-
tandem mass spectrometry [27]. We have successfully used this method to identify specific antigens in circulating ICs (CIC-antigens) in serum or
cerebrospinal fluid recovered from subjects with autoimmune diseases,
infectious diseases, and cancers, as well as those who are liver trans-

plant recipients [27–31].

In the present study, we applied immune complexome analysis to SP

and FF collected from infertile couples undergoing assisted reproduc-
tion. The goal of this analysis was to comprehensively identify IC-antigens, with the intent of identifying those IC-antigens specific for in-

fertile males and for infertile women with endometriosis. Formation of

ICs between the specific antigens and their corresponding auto-

antibodies might affect the physiological functions of the antigens, possibly leading to male and female infertility. Additionally, IC for-

mation and deposition on tissues is known to stimulate inflammatory

processes via the action of the complement system. It is hoped that the

present study may lead to elucidation of the pathogenesis of male in-

fertility and endometriosis involving immune abnormality, and to the
development of new therapies to treat these fertility challenges.

2. Materials and methods

2.1. Patients

All samples were collected from infertile couples undergoing as-

sisted reproduction technology (ART) at Nagasaki University Hospital;

written consent was obtained from all participating patients. Infertility is
defined as the couple who suffer from the failure to achieve a clinical

pregnancy after 12 months or more of regular unprotected sexual in-
tercourse. Before ART, male subjects received semen analysis and fe-

male subjects received the test for tubal patency by hysterosalpingo-

graphy or laparoscopy, ovarian function by hormonal analysis and

serial transvaginal ultrasonography, pelvic pathology, such as uterine

and ovarian tumors, by ultrasonography and/or MRI. The couples with

azoospermia or primary ovarian insufficiency were excluded. This

study was performed according to Helsinki Declaration and was ap-

proved by the Nagasaki University Hospital Ethics Committee

(Research Ethics Committee Approval No. 16020804).

2.2. SP

Forty-two semen samples were collected. After complete liquefa-

ction, an aliquot of the ejaculate was employed for semen analysis using a

Makler counting chamber (Irvine Scientific, Santa Ana, CA). Semen

volume (mL), sperm concentration (10⁶/mL), and motility (%) were meas-

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volume (mL), sperm concentration (10⁶/mL), and motility (%) were recorded and classified according to the WHO guidelines [32]. Subjects

were divided into three groups: the oligozoosperma group (n = 6), which consisted of male subjects with sperm concentrations lower than

15 × 10⁶/mL without regard for sperm motility; the asthenozoos-
permia group (n = 8), which consisted of male subjects with sperm concentrations lower than 15 × 10⁶/mL without regard for sperm motility; the asthenozoosperma group (n = 8), which consisted of male subjects with sperm motility < 40% but with normal sperm concentrations (> 15 × 10⁶/

mL); and the normal control group (n = 28), which consisted of males

with normal sperm concentration and motility (> 15 × 10⁶/mL with motility > 40%). In these reports, endometriosis shows

findings imply that the local

fertility and endometriosis involving immune abnormality, and to the
development of new therapies to treat these fertility challenges.

Endometriosis is a chronic pelvic inflammatory disease and one of

the major causes of female infertility. Endometriosis is characterized by

the presence of ectopic endometrial tissue outside of the uterine cavity,

and is manifested by chronic local inflammation in the pelvis. As with

spermatogenic dysfunction, the pathogenesis of endometriosis is enig-
matic. However, local immunity in the female pelvis also may be in-
involved in the pathogenesis of endometriosis-related infertility. The

mechanisms of endometriosis-related infertility vary, and include

peritoneal adhesion, dysfunctional uterotubal motility, disturbed folli-
culogenesis, and detrimental effects on spermatozoa [14,15]. Ovarian

endometriotic lesions (endometriomas) are one of the main disease

phenotypes, and local inflammatory reactions surrounding en-
dometriomas may affect folliculogenesis and the process of oocyte

maturation [14]. In fact, the ovary affected by endometriomas may

show fibrosis and altered folliculogenesis, effects that may result from

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couple with female factor infertility. In oligozoospermia group, all couple were primary infertility and there were no female partners with infertility factors. In asthenozoospermia group, six subjects were primary infertility and two subjects were secondary infertility, and there were three couples with female factor infertility, such as endometriosis (n = 2) and ovulatory dysfunction (n = 1). In normal control group, 24 subjects were primary infertility and four subjects were secondary infertility, and there were 15 couples with female factor infertility, such as tubal factor (n = 6), ovulatory dysfunction (n = 5), endometriosis (n = 3), and woman with anti-centromere antibody (n = 1).

2.3. FF

Fifty-eight samples were collected; the subjects were divided into two groups. The endometriosis group consisted of 10 women who had been diagnosed as having one or more endometriomas at a previous operation. At the time of surgery, cystectomy was performed in six subjects, drainage and cystic wall ablation were performed in three subjects and hemilateral cystectomy and contralateral ablation were performed in one woman with bilateral lesions. The average period from surgery to oocyte retrieval was 2.8 ± 4.1 years (mean ± standard deviation). The other women lacked subjective and objective clinical symptoms of endometriosis and were designated as a control group (n = 48). Although all subjects received pelvic examination and transvaginal ultrasonography before ART to rule out the pelvic pathology, 15 (31%) of them had been undergone pelvic surgery for indications other than endometriosis, and they were confirmed not to have the disease. At the time of transvaginal oocyte retrieval, FF was collected from the first punctured follicle. After transferring the egg to the culture medium, FF was centrifuged at 2.8 × 10^5 × g for 5 min and an aliquot (10 μL) of the supernatant was used for immune complexome analysis.

3. Experimental

3.1. Immune complexome analysis

ICs in SP or FF were collected using Proceptor™-sepharose beads. An aliquot (40 μL) of each bead type was incubated with 10 μL of pooled human serum diluted with 90 μL phosphate-buffered saline (PBS) for 30 min with gentle mixing. The beads were pelleted by 1 min of centrifugation and the supernatant was removed with a pipette. The beads were washed three times with 500 μL PBS/wash. Washed beads were suspended in 50 μL of 10% PBS and papain solution (0.04 M EDTA, 0.04 M L-cysteine) and incubated at 37 °C for 30 min. Then, 50 μL of 0.06 M iodoacetic acid in PBS was added to quench the papain digestion. Next, we added 100 μL of 10 mM dithiothreitol and further incubated the sample at 56 °C for 45 min. Then, 100 μL of 55 mM iodoacetic acid was added, and the mixture was incubated in the dark at room temperature for another 30 min. Trypsin in 0.05% acetic acid was added to yield a final concentration of 0.5 g of trypsin/L, and the mixture was incubated overnight at 37 °C. An aliquot (12 μL) of 10% TFA in water was added to the mixture to quench the digestion. The beads were pelleted by 1 min of centrifugation; the resulting supernatant (approximately 400 μL) was recovered, vacuum-reduced to a volume of approximately 80 μL, and stored at 4 °C pending subsequent analysis by nano-LC-MS/MS. The peptide mixture (1 μL) was injected into an LC-electrospray ionization (ESI)-MS/MS instrument (Q-Exactive, Thermo Fisher Scientific, Waltham, MA, USA) equipped with EASY-nLC™ 1200 system consisting of a nano LC pump) and an autosampler was used for analysis. Peptides were deionized and were concentrated on pre-column (AccepMap™ 100, 75 μm × 2 cm, nano Viper, C18, 3 μm, 100 Å, Thermo Fisher Scientific), and were subsequently separated on a nano-LC column (C18, 75 μm i.d. × 125 mm, 3 μM particle, 100 Å pore size, Nikkyo Technos, Tokyo, Japan) and ion-sprayed into MS with a spray voltage of 1.5 kV. The separation was performed by using the mobile phase A (0.1% formic acid) and mobile phase B (0.1% formic acid in 90% acetonitrile), employing a gradient elution from 5% to 33% mobile phase B in 100 min, and 100% mobile phase B held for 10 min. MS/MS data were extracted using Proteome Discoverer 1.3.1.339 (Thermo Fisher Scientific). Spectra were searched against sub-databases from the public non-redundant protein database of UniProt Knowledgebase (human, 2015.01.29 download) with the following search parameters: mass type, monoisotopic precursor and fragments; enzyme, trypsin (KR); enzyme limits, full enzymatic cleavage allowing up to two missed cleavages; peptide tolerance, 10 ppm; fragment ion tolerance, 0.8 Da; ion and ion series calculated, B and Y ions; static modification, C (carbamidomethylation); and differential modifications, M (oxidation), N, and Q (deamidation). All the results were obtained by triplicate analyses. All the peptides and proteins found in the first, second and/or third analysis were counted in the numbers of identified peptides and proteins. The procedure has been described in detail in our previous publication [33].

3.2. Statistical analysis

Statistical analysis was performed using Stat Mate V software (ATMS, Tokyo, Japan); the significance level was defined as a p value < .05. An F test was used to test the normality of distributions. Continuous variables that exhibited skewed distributions by the F test were analyzed using a two-tailed Kruskal-Wallis H test with post-hoc Dunnett’s test. Continuous variables that exhibited normal distributions by the F test were analyzed using a two-tailed one-way analysis of variance (ANOVA) with post-hoc Tukey-Kramer test. For female patients, age, serum anti-Müllerian hormone (AMH), and the number of retrieved oocytes were analyzed using a Mann-Whitney U test. Fisher’s exact test for parity was used for comparisons between two groups.

4. Results

4.1. Clinical backgrounds of the male subjects provided seminal plasma

Age and the results of semen analysis in the male subjects are summarized in Table 1. There was no statistically significant difference in age or semen volume among the three groups. Consistent with the group-assignment criteria, sperm concentrations in the oligozoospermia group were significantly lower than those in the other groups (p < 0.05 versus asthenozoospermia, p < 0.001 versus normal semen analysis). Similarly, there was a statistically significant difference in sperm motility, between the asthenozoospermia group and the normal group (p < 0.001). We also detected a significant difference in sperm motility between the oligozoospermia and normal groups (p < 0.01).

Table 1

<table>
<thead>
<tr>
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<th>Oligozoospermia (n = 6)</th>
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<th>Normal (n = 28)</th>
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<tr>
<td>Age (years)</td>
<td>41 ± 6</td>
<td>39 ± 3</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>Sperm volume (mL)</td>
<td>2.8 ± 1.8</td>
<td>2.8 ± 1.6</td>
<td>3.4 ± 1.4</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>4 ± 1.3</td>
<td>85 ± 48</td>
<td>104 ± 47</td>
</tr>
<tr>
<td>(10^6/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motility (%)</td>
<td>37 ± 12(^a)</td>
<td>28 ± 8(^b)</td>
<td>58 ± 13</td>
</tr>
</tbody>
</table>

Footnote: Data are presented as mean ± SD (standard deviation). Age, semen volume, sperm concentration, and motility were compared among the three groups. After an F test was used to check the variance among the groups, age, semen volume, and motility were analyzed using a two-tailed Kruskal-Wallis H test with a post-hoc Tukey-Kramer test.

\(^a\) p < 0.001 versus normal semen.

\(^b\) p < 0.05 versus asthenozoospermia.
Four asthenozoospermia-specific antigens (dual specificity testis-specific protein kinase 2 (also known as TESK2), probable E3 ubiquitin-protein ligase HERC1, uncharacterized protein KIAA1109, protein arginine N-methyltransferase 7, and ATP-binding cassette subfamily F member 1) were detected in SP from six of eight subjects with asthenozoospermia; these antigens were not detected in SP from the other two groups (Table 4). Each antigen was detected at a frequency of 25% within subjects with asthenozoospermia without a specific pattern. SP from two subjects retained three disease-specific antigens; SP from four patients retained one specific antigen; and SP from two patients did not retain any of these specific antigens. There was no specific pattern regarding the number of antigens present per subject, nor did the presence of various antigens appear to relate to the severity of asthenozoospermia (data not shown).

4.3.2. FF
Although the majority of 327 antigens identified in FF were present both in FF from subjects with endometriosis and in FF from those without endometriosis, eight antigens were detected only in the endometriosis group; these eight antigens were not detected in FF from the control group (Table 5). These specific antigens (fibroblast growth factor receptor 1 (also known as FGFR1), probable ubiquitin carboxy-terminal hydrolase FAF-Y (also known as Deubiquitinating enzyme FAF-Y), interleukin-6 receptor subunit beta (also known as gp130), sentrin-specific protease 1, centlein, Neutralized-like protein 4, apollipoprotein B receptor, and WSC domain-containing protein 1) in FF were detected in six of ten subjects. Each antigen was detected at a frequency of 20% among subjects with endometriosis; no specific pattern was observed. FF from one subject each retained five specific antigens, four specific antigens, and three specific antigens; FF from three subjects retained one of these antigens, while those from the remaining four subjects did not harbor any of these specific antigens. There was no specific pattern regarding the number of antigens present per subject, nor did the presence of various antigens appear to relate to the severity of endometriosis (data not shown).

5. Discussion
In this study, we demonstrated for the first time (to our knowledge) that disease-specific ICs are formed in the local body fluids of the reproductive tracts, such as SP and FF, obtained from infertile males and females, when assessed by proteomic immune complexome analysis. ICs are formed by the binding of immunoglobulins to self and non-self antigens to promptly recognize autoantigens or prevent the spread of non-self antigens. When excessive numbers of ICs are produced, the complexes may induce inflammation and tissue damage via activation of complement. In addition, the deposition of ICs in tissues can cause fibrosis, atrophy, and dysfunction due to type-III hypersensitivity, endothelial dysfunction, and tissue remodeling [35]. Moreover, IC-associated antigens (proteins) may lose their function due to IC formation. Thus, comprehensive analysis of ICs and their antigens may facilitate the definition of the pathogenesis of disorders relevant to immune responses and inflammation.

In this study, we demonstrated for the first time (to our knowledge) that disease-specific ICs are formed in the local body fluids of the reproductive tracts, such as SP and FF, obtained from infertile males with spermatozoal dysfunction and females with endometriosis, when assessed by proteomic immune complexome analysis. Immune response and inflammation in the local environment may be involved in the pathogenesis of both disorders. To our knowledge, most of the disease-specific antigens identified in this study had not previously been reported to be associated with the respective disease; nonetheless, some of these antigens may correspond to the disease pathology. The specific antigens detected in SP may function as components of BTB, DNA repair machinery, or sperm nuclear envelopes. In contrast, the proteins specifically detected in FF are known to be related to inflammation.
Interestingly, none of the detected IC-antigens were shared among the oligozoospermia, asthenozoospermia, and endometriosis groups. These results may reflect the specific role of ICs in each disorder, implying that the analysis of ICs may contribute to expanding our knowledge of specific diseases.

Several papers have suggested an association between spermatogenic dysfunction and autoimmune response against sperm [1,3,5,6]. For instance, the BTB is formed by Sertoli cells in the seminiferous epithelium and plays an important role in maintaining a microenvironment (notably, an immunoprivileged compartment) that is suitable for spermatogenesis [1]. However, the BTB can be disrupted by inflammation; therefore, identification of specific BTB autoantigens...
may be crucial for understanding the pathological processes of BTB disruption [36]. In this context, among the nine disease-specific antigens identified in the SP of the oligozoospermia and asthenozoospermia groups, zyxin and TESK2 are known components of the BTB [37,38]. Production of antibodies against these proteins, as suggested by the presence of corresponding ICs in the present study, may lead to antigen-specific inflammation and BTB disruption.

SPANX-D, which was detected in half of SP specimens derived from oligozoospermia subjects, and the probable E3 ubiquitin-protein ligase HERC1 (HECT-type E3 ubiquitin transferase HERC1), which was specifically detected in the asthenozoospermia group, are proteins involved in DNA damage repair [39–41]. IC formation may result in disorganized sperm production due to accumulation of DNA damage and genome instability by loss of the functions of these proteins.

Among the eight specific antigens found in FF of subjects with endometriosis, several proteins might be involved in the pathogenesis of endometriosis, such as gp130, Deubiquitinating enzyme FAF-Y, and FGFR1. These proteins are known to regulate local inflammation, inflammasome formation, or the epithelial-to-mesenchymal transition (EMT) [42–45]. FF may be involved in the growth and maintenance of superficial ovarian endometriomas and peritoneal lesions [24]. Loss of function of specific antigens (which would result from excess formation of ICs) may render the local pelvic micro-environment favorable to the progression of endometriosis. IL-6 is a pro-inflammatory cytokine and may be involved in endometriosis-associated infertility [46]. The formation of ICs that include the IL-6R subunit beta (also known as gp130), which inhibits the pro-inflammatory trans-signaling cascade of IL-6 by binding to the complex formed by IL-6 and sIL-6R [42,47], might lead to activation of trans-signaling and exacerbation of inflammation.

On the other hand, altered folliculogenesis caused by destruction of normal ovarian cortical structures may be one of the causes of endometriosis-related infertility. Fibrotic changes in the ovarian cortex are associated with decreased follicular density and enhanced follicular recruitment and atresia [17,18]. Dysregulation of the inflammasome and EMT caused by the formation of ICs including Deubiquitinating enzyme FAF-Y and FGFR1, respectively, may be associated with exacerbation of inflammation and fibrosis [48,49]. Deubiquitinating enzyme FAF-Y may function as a polyubiquitin hydrolase that counteracts the activity of TRIM33, an E3 ubiquitin-protein ligase [43]. TRIM33 is essential for activation of the NLRP3 inflammasome [44]. FGFR1 has been shown to affect myofibroblast differentiation by inhibiting signaling by TGF-β1 and the FGF-1 ligand, events that lead to reversion of the EMT [45]. In terms of inflammation and fibrosis, IC formation would result in activation of the complement system, a process that is known to be involved in the pathogenesis of endometriosis [50].

6. Conclusions

We comprehensively identified the constituent antigens of ICs in SP and FF via immune complexome analysis. Among the 391 and 327 human antigens detected in SP and FF, nine and eight antigens were found to be specific to subjects with spermatogenic dysfunction (four antigens for oligozoospermia and five antigens for asthenozoospermia) and ovarian endometriosis, respectively. Several antigens and the corresponding proteins coincide with known disease characteristics and may be involved in the pathogenesis of male and female infertility. Other specific antigens that lack known functions but were detected in SP and FF may have unknown roles in infertility. Immune complexome analysis may be a useful technique for revealing disease pathogenesis and may contribute to the development of new treatment strategies for reproductive dysfunction. However, our results, which were derived from a relatively limited number of subjects in the present work, will need to be confirmed in large-scale studies. Additionally, the exact relationship between specific antigens and male and female infertility related to spermatogenesis and endometriosis will need to be examined.

We expect that further analysis of these disease-specific antigens may provide a better understanding of the pathogenesis of both conditions.

Aknowledgement

This research was supported in part by the Grants-in-Aid for Scientific Research (grant no. 18K09294 and 16K20197 to M.K. and N.M.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Disclosure/Conflict of interest

No potential conflicts of interest were disclosed by all the authors.

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