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IDENTIFICATION METHODS AND CHARACTERISTICS OF ANTIBODIES FOR HSP60 IN PREGNANT WOMEN

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Abstract

Hsp60 comes among the first proteins synthesized during embryogenesis and it is required for the development of embryo. High serum level of anti-Hsp60 antibodies is a poor sign as such antibodies may inhibit embryonic development and affect pregnancy outcome (induction of premature labour). The study goal was to detect and characterize anti-Hsp60 autoantibodies in pregnant women. The anti-Hsp60 antibodies' level and their ability to recognize target antigens were detected in serum of pregnant women using the enzyme immunodetection and Western blotting technique respectively. There have been examined 170 patients, the control group of which is represented with 20 clinically healthy pregnant women and another group contains 150 pregnant women with complicated anamnesis. The obtained recombinant human Hsp60 was used as an antigen. Anti -Hsp60 positive serum was found in 7.7% women from the control group and 14% women from the complicated anamnesis group. There hasn't been established any statistically significant difference between the anti-Hsp autoantibodies level (by mean average) in the studied groups. Pregnant women with high serum levels of anti-Hsp autoantibodies were more often previously diagnosed with reproductive dysfunctions (spontaneous abortions, missed miscarriage –1.5 times), as well as previous operative interventions (tonsillectomy- 7 times and appendectomy- 1.8 times) compared to pregnant women with low content of the studied antibodies. Western blotting technique showed high reactivity properties for Hsp60 exhibited by the serum of pregnant women with previous miscarriage or missed miscarriage caused by pelvic diseases. The study of the serum immune reactivity for Hsp60 in pregnant women may turn out to be an additional pregnancy outcome prognostic test.

Key words: Pregnancy, Hsp60, Antibodies for Hsp60.

Introduction

The heat shock proteins, HSPs or molecular chaperones were primarily characterized as agents of cellular response onto stress (like infection, heavy metals' influence, ischemia, hypoxia, aminoacid deficiency, psycho-emotional stress, hormonal stress, etc.). Nowadays HSPs are considered to be extremely important for the inborn and adaptation immunity [11].

Hsp60 is an evolutionally conservative chaperone/ chaperonin of mitochondrial origin, Hsp10 in mitochondria uses co-chaperone and provides for the correct positioning of the cellular proteins, in case of stress it prevents their faulty aggregation and folding. Hsp60 is also detected in cellular cytoplasm where it exhibits antiapoptotic function. Under the stress conditions Hsp60 is displayed onto cellular surface and secreted into intracellular space [6].

It has been established that chaperonin is a target antigen in case of autoimmune diseases (atherosclerosis, type 1 diabetes, etc.), and the Hsp-60-reactive T- and B-cells provide for the immune response in infectious diseases. Hsp60 is characterized by immunoregulatory properties and it may produce both pro- and anti-inflammatory effects, due to the microenvironment in the target organ, which makes it an important homeostatic antigen[13].

Hsp60 comes among the first proteins synthesized during embryogenesis and it is required for development of embryo [9]. The study proved direct influence of anti-Hsp60 antibodies onto the murine embryos *in vitro* [10]. The increased Hsp60 content is detected in early pregnancy period (weeks 7-11 of pregnancy) in maternal placenta. This may present a potential target for cross-reacting antibodies for microbial Hsp60 as well as lymphocytes sensitized by the Hsp60, which would cause immune rejection of the embryo. The study established that the Hsp60 placental level didn't differ in healthy pregnancy and early abortion pregnancy. Though, the immune complex "Hsp60-anti-Hsp60 antibody" were revealed only in placenta of women with early miscarriage. Promotion of anti-inflammatory cytokines synthesis by such immune complexes may negatively affect pregnancy outcome (induction of the premature labour) [17].

The aim of the study was to detect and characterize anti-Hsp60 autoantibodies in pregnant women for evaluating possibility of their use as an additional test for pregnancy development prediction.

Study methods and materials.

Study Object: The study includes the data of laboratory and instrumental examination of 170 pregnant women (according to the Decree of Ministry of Health of Ukraine № 417, issued on 15.07.2011 "On management of

outpatient obstetrical and gynecological supervision in Ukraine"), which included 20 clinically healthy pregnant women (control group) and 150 pregnant women with complicated anamnesis (main group).

The serum IgG level of anti-Hsp60 antibodies was detected using the enzyme immunodetection method in various versions [4].

The antigen was diluted in phosphate-buffer saline (PBS 140 mM of NaCl, 50m of phosphate buffer pH 7.2) and in concentration of 10 mkg/ml it was introduced (volumed 100 mkl) into the cavities of polystyrene 96-well plate, then incubated during the night under 4° C for further protein absorption onto the plastics. The control cavities were filled with BSA (concentrated as 10 mkg per liter) dissolved in PBS. To remove the antigen which wasn't bound, the plates were thoroughly cleaned with the solution of PBS with 0.1% twin-20 (PBS-T). To prevent non-specific binding of serum proteins to adsorbed protein 100 mcl of PBS-T were introduced, to be further incubated for 1 hour under 37°C. After cleaning the plates with PBS-T, solution was removed, and the cavities were filled with the serum of pregnant women, diluted as 1:100 (repeated three times). The plates were incubated for 18 hours under 4°C. After completing incubation, the solution was removed and plates were cleaned with PBS-T solution.

Then secondary antibodies of the rabbit for human IgG marked with chromium peroxidase ("Promea" production) were introduced and incubated for an hour under 37°C. After cleaning the plates, the cavities were filled with the substrate for chromium peroxidase ABTC, concentrated as 0.5 mkg/ml in 50 mM of the citrate buffer, pH 5.0, with 0.05% hydrogen peroxide *solution*.

15 minutes after incubation of the plates under 37°C, optic density was estimated (wave length equals 405nm) using the "Multiscan" equipment («Titertek», the UK). Positive control was presented with high reactive serum with polyclone antibodies for Hsp60, obtained according to L. Sidorik et al. [15], negative control presented with the low reactive serum of clinically healthy donors.

Recombinant human protein Hsp60 was used as antigen, it was obtained earlier. The recombinant protein Hsp60 was obtained and purified according to the introduced method [2]. We defined the serum as an antibody-positive one when its optic density (diluted as 1:100) exceeded mean average of optic density of the serum obtained from the control group by two standard deviations (m+2sd).

Protein concentration detection was performed according to Bredford [5].

Electrophoresis of recombinant human Hsp60 was performed in 12% polyacrylamide gel under denaturant conditions, according to U. Laemmli [8].

Western blotting technique (immunoblotting)

Protein was being transferred from gel onto nitrocellulose membrane using protein electrotransport during 40 minutes under 90V voltage, with buffer composed of 200mM of glycine, 25 mM of tris-HCl (pH 7.4) and twenty-percent methanol. The membrane was cut onto bands and it was being blocked with five percent solution of defatted milk in PBS-T buffer for one hour under the room temperature. After this the bands were being incubated under the presence of individual serum, diluted as 1:100 in PBS-T, during the night under 4ºC. The membrane bands were cleaned with PBS-T from antibodies which weren't bound, to be further incubated for one hour under the room temperature together with secondary antibodies, after which they were cleaned. To detect the specific binding of secondary antibodies the membrane was being kept in ECL-reactive chemical for 5 minutes and then visualized using the transilluminator ChemiDoc XRS+.

The statistical processing of the data was performed using the software STATISTICA 8.0 (Stat-Soft, 2007, the USA). To compare group samplings Mann-Whitney U-test was used, some results presented as mean averages with noted square deviations from the mean.

Study results. Discussion

We studied possible immune reactivity of the serums taken in pregnant women for eukaryotic chaperonin Hsp60 using enzyme immunodetection and Western blotting method. Such combination of methods allows detecting both antibodies' level and their potential possibility to bind with the target antigen. Using the enzyme immunodetection method we revealed anti-Hsp60 positive serum in 7.7 % of pregnant women from the control group and 14.0% of pregnant women with complicated anamnesis. There hasn't been detected a statistically significant difference between the anti-Hsp60 auto-antibodies' level (by mean average data) in pregnant women of the main group and those of the control group (0.177 ± 0.118 versus $0.134 \pm 0{,}097$ units of optic density, $p>0.05$). The incidence of pathologies in pregnant women with positive anti-Hsp60 serum was compared to the incidence in pregnant women with negative anti-Hsp serum, the results being presented in the table. The study revealed that the main group of pregnant women with high anti-Hsp60 human antibodies serum level was characterized with higher anamnesis reproductive dysfunctions data (spontaneous miscarriage, missed miscarriage- 1.5 times higher) and operative interventions (tonsillectomy –7 times and appendectomy 1.8 times) compared to pregnant women with low serum level of the antibodies. The results of pregnant women serum immunoreactivity study using the Western blotting analysis method are represented in picture 1.

It should be noted that only one serum sample of the control group was high-reactive for the studied antigen. The serum of pregnant women with previous miscarriage related to pelvic diseases was high-reactive in Western blotting. The Western blotting parameters sometimes didn't coincide with the enzyme immunodetection data (low level of antibodies obtained by enzyme immunodetection and high reactivity of the studied antibodies in Western blotting). The ability of recognizing target antigen (human Hsp 60), up to our mind, is a more important characteristic and it may evidence about potential ability of the antibodies to bind with endogenous Hsp60. The study indicated that the anti-Hsp60 antibodies may mediate complement-dependent cytotoxicity, cause cytolysis or apoptosis of the cells which express Hsp60 onto their surface. The antibodies may also bind blood plasma-circulating endogenous Hsp60 and form immune complexes with pathological effect onto the cells and tissues [6].

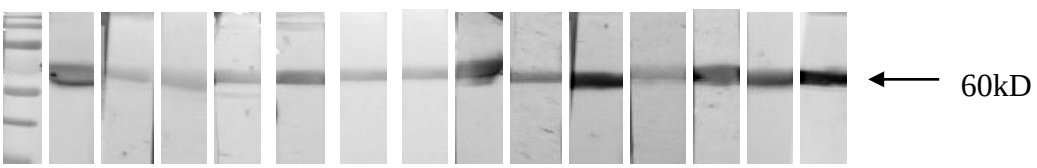
Anti-Hsp 60 antibodies with ranging specificity are detected in serum of healthy women and their level increases in case of various diseases [12]. High level of anti-Hsp60 antibodies should be considered as a biological marker of morbidity, chronic diseases, autoimmune diseases, signs of cardio-vascular diseases and their severity, risk factor of atherosclerosis, cancerogenesis marker, etc. [14, 16].

Before this we detected relation between increased level of antibodies for the microbial homologue of human Hsp60 which was chaperonin of *E.coli* (GroEL) and the reproductive dysfunctions (tubal infertility, miscarriage) (Pearson coefficient, K=0,475; mean average relation) [3]. According to our previous studies, the anti-GroEl antibodies' level ranged within control figures in 92.86% of all pregnant women. Pregnant women with anti-GroEl positive serums were noted with changed clinical data (such as leukocytosis, increased ESR), risk of miscarriage, and previous premature labour and miscarriage in anamnesis. The performed earlier study of anti-GroEl antibodies in serum of pregnant women, the fetuses of which had prenatally been diagnosed with critical inborn heart defects, didn't detect statistically significant difference in studied antibodies in examined pregnant and clinically healthy women (blood donors) according to mean average figures[1]. Literature analysis showed that level of anti-Hsp antibodies in the women whose children were born with anatomical defects didn't reliably differ from the control group (pregnant women with healthy pregnancy development) [7]. Low level of antibodies for human Hsp60 detected in 92.3% of pregnant women from control group and 86% of pregnant with complications in anamnesis agree with the results obtained before as well as the literature data. Though, high reactivity of serum established by Western blotting is an unfavorable sign for pregnant women. As it was mentioned before, anti-Hsp antibodies may inhibit development of the embryo as well as negatively affect pregnancy outcome (premature labour) [9, 10]. This is why pregnant women

the serum of whose is characterized with high reactivity for human Hsp60 require for thorough clinical and laboratory examination.

Table-1: Frequency of pathologies detected in pregnant women with high serum level of anti-Hsp60 auto antibodies.

	Pregnant women with high serum level of anti-Hsp60 autoantibodies	Pregnant women with low serum level of anti-Hsp60 autoantibodies
Spontaneous miscarriage, missed miscarriage in anamnesis	31.8%	21.7%
Tonsillectomy in anamnesis	14.3%	2.3%
Appendectomy in anamnesis	23.8%	13.28%
Inflammation of pelvic organs	66.67%	53.9%
Inflammatory gastro-intestinal diseases in anamnesis	33.3%	25.78%
Inflammatory genitourinary diseases in anamnesis	19.0%	17.97%
Diseases of the thyroid gland	4.76%	12.5%
Varicose veins	4.76%	3.12%



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Picture 1. Immunoreactivity (recognizing target antigens) of serum obtained from clinically healthy pregnant women and women with disordered reproductive functions (spontaneous miscarriage or missed miscarriage in anamnesis) detected by Western blotting.

1. Mixture of protein markers with different molecular weight.
2. Positive serum (Polyclone anti-Hsp60 antibodies)

3-8. Serum of clinically healthy pregnant women (diluted as 1:100)

9-15. Serum of pregnant women with disordered reproductive functions (spontaneous miscarriage or missed miscarriage in anamnesis) (diluted as 1:100).

Human recombinant protein Hsp60 was used as an antigen (3 mkg for a cavity).

Conclusions

The ability of recognizing target antigen (human Hsp 60) is a more important characteristic and it may evidence about potential ability of the antibodies to bind with endogenous Hsp60. The study indicated that the anti-Hsp60 antibodies may mediate complement-dependent cytotoxicity, cause cytolysis or apoptosis of the cells which express Hsp60 onto their surface. The antibodies may also bind blood plasma-circulating endogenous Hsp60 and form immune complexes with pathological effect onto the cells and tissues.

So, the obtained results evidence that combination of enzyme immunodetection and Western blotting is more appropriate as an additional test regarding the study of anti-Hsp60 antibodies.

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