CHAPERON HSP–60 AS AUTOANTIGEN IN DEVELOPMENT OF DYSHORMONAL BREAST DISEASES

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ШАПЕРОН HSP–60 КАК АУТОАНТИГЕН ПРИ РАЗВИТИИ ДИСГОРМОНАЛЬНЫХ ЗАБОЛЕВАНИЙ МОЛОЧНОЙ ЖЕЛЕЗЫ

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Recombinant Hsp-60 chaperon was isolated from the lyzate of *E.coli* cells transformed with plasmid pT-GroESL, purified by gel-filtration and ion exchange chromatography and used as antigen in the study of autoimmune processes in patients with dyshormonal breast diseases (DBD). It has been shown that the sera of 80% of DBD patients were anti-Hsp-60 positive and the level of anti-Hsp-60 antibodies in the sera of DBD patients was significantly higher ($p < 0.05$) that that in the sera of healthy donors. The highest levels of anti-Hsp-60 antibodies were registered in the sera of the DBD patients with simultaneous cancer processes.
***Key Words*: chaperon, dyshormonal breast diseases, breast cancer.**

Рекомбинантный шаперон Hsp-60 выделен из лизата клеток *E.coli,* трансформированных плазмидой pT-GroESL, очищен путем гель-фильтрации и ионообменной хроматографии и использован как антиген при изучении аутоиммунных процессов у пациентов с дисгормональными заболеваниями молочной железы (ДЗМЖ). Установлено, что сыворотка крови 80% пациентов с ДЗМЖ является анти-Hsp-60 положительной, причем количества анти-Hsp-60-антител в сыворотке крови пациентов с ДЗМЖ значительно превышают таковые у здоровых доноров. Наибольшие количества анти-Hsp-60-антител выявлены в сыворотке крови пациентов с ДЗМЖ и одновременным наличием других опухолевых процессов.
***Ключевые слова*: шаперон, рак молочной железы, дисгормональные заболевания молочной железы.**

It is well known that the development of dyshormonal breast diseases (DBD) is tightly linked to risk of oncogenesis in women in menopausal and postmenopausal period. According to the data of some authors the risk of the development of breast cancer may be 37–fold higher in DBD patients than in healthy subjects [1]; the relative risk is determined by morphologic peculiarities of hyperplasia and is genetically based. The maximal frequency of DBD and more than 60% breast cancer cases are registered in patients at the age when ovary involution begins [1–5]. The tight cooperation of immune and endocrine systems in development of pathologies of endocrine organs and the involvement of autoimmune processes in those disturbancies is well recognized now [6].

At the same time the molecular mechanisms of DBD development via autoimmune pathology remained poorly studied yet. The main research should be aimed on the search of autoantigens–targets expressed in the affected organs; the characterization of such antigens will help to understand ethio– and pathogenetic mechanisms of DBD development and design new approaches for DBD diagnosis and therapy.

The majority of autoimmune diseases accompanied by the death of cells in targeted organ with the development of fibrosis and sclerosis foci are characterized by wide spectrum of autoantibodies to nuclear and cytoplasmic components [7–10]; among the last ones chaperons (Hsp) occupied the special place. Chaperons respond for protein folding, protein association in oligomeric complexes, and the import of protein precursors to intracellular compartments [11]. A lot of data evidence that the wrong folding of proteins forms the molecular basis of some human diseases [12].

Earlier it has been shown that bacterial chaperon Hsp–60 (isolated from *Salmonella, Yersinia*, and *Chlamydia)* is a highly concervative protein and has high homology with chaperons from other species, including mammals and men [13]. It was shown that upon infection caused by *Chlamydia* the induction of anti–bacterial Hsp–60 immunity provokes autoimmune answer against own Hsp–60 of the host [14]. It is demonstrated also

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Abbreviations used: DBD — dyshormonal breast diseases; Hsp — chaperon.

that DBD development is often accompanied by increased level of autoantibodies against DNA–Topoisomerase I, an enzyme determining the folding, integrity and functioning of DNA [15]. This fact reflects the possible involvement of autoimmune mechanisms in DBD development. One may suppose that the system of protein folding could be affected, too. The present research was aimed on the study of the expression of Hsp–60 chaperon as autoantigen in regard to the occurrence of benign dysplasia and benign tumor of the breast.

MATERIALS AND METHODS

The samples of blood serum were obtained from 25 patients with benign DBD in perimenopause (5 patients from this group (cases 1, 4, 23–25) had simultaneous tumor processes — fibromyoma of uteri, hyperplasia of endometrium and nodular thyroid pathology). DBD cases included: 7 cases of diffuse fibroadenomatosis, 4 cases of fibrocystic hyperplasia, 3 cases of fibrohyperplasia, 2 cases of adenohyperplasia, 2 cases of fibroadenoma, 1 case of galactocele, 2 cases of nodular fibroadenoma, 2 cases of involutive alteration of the breast, 2 cases of the mixed forms of dyshormonal disease. The sera of 32 healthy women of the same age were used as a control. The recombinant chaperon Hsp–60 (GroEL) was used as positive control.

The purification of Hsp–60 chaperon. The protein was isolated from lyzate of *E.coli* cells transformed with plasmid pT–GroESL. pT–GroESL was constructed on the base of pACYC vector and contains coding sequence GroEL and GroES genes under T7 promoter, p15b replicon and a marker of chloramphenicol–resistance. The cell line transformed with pTGroESL plasmid is overexpressing chaperons (the yield of chaperons with molecular weight 60 kDa in the soluble fraction was 30–50% of total cell proteins).

The purification was performed in 3 stages: precipitation with saturated ammonium sulphate solution, gel–filtration, ion exchange chromatography. Gel–filtration was carried out on Sephacryl S–300 column (1 x 26 cm) in the buffer A (10 mM Tris–HCl, pH 8.0, 50 mM NaCl). Fractions (1 ml) containing shaperons were collected and applied on DEAE–Toyopearl 650M column (1 x 6 cm) equilibrated with buffer A. The elution of proteins was performed in NaCl gradient in the range 50–500 mM. Chaperon–containing fractions were collected, dialyzed against buffer A and concentrated by centrifugation in Bio–Rad Unisep Ultracent centricons at 4 °C. The protein was dialysed against buffer A containing 30% of glycerol and was stored at –20 °C. For chaperon identification the immunoblotting technique was applied using polyclonal anti–Hsp–60 antibodies. Polyclonal antibodies were obtained according to the method [16] by immunization of rabbits with microquantities of antigen (< 25 μg/ animal). The affine column was prepared as described in [16]. The purity of the proteins was estimated by Laemmly electrophoresis [17]. The concentration of proteins was measured by method of Bradford [18].

Immunolinked immunosorbent assay (ELISA) was performed by modified method [19]; taking to account low affinity of autoantibodies the plates with the sera of patients were incubated overnight at 4 °C.

Western–blot analysis was carried out according to [20] using recommendations for ECL–system (Pierce, UK); the blots were incubated with sera samples overnight at 4 °C. Statistical analysis was performed with the use of program "STATISTICA for Windows 5.0" and Student's *t*–test. The values $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Hsp–60 protein was isolated from cell lyzates by gel–filtration and ion–exchange chromatography on DEAE–Toyopearl 650M (Fig. 1). The purity of Hsp–60 protein was evaluated by Laemmli electrophoresis in denaturing conditions (Fig. 2). The data obtained demonstrated that the applied scheme for purification of Hsp–60 protein allows to obtain the pure stable protein in the short terms (in a week). The specificity of purified Hsp–60 antigen was determined by immunoblotting using commercial anti–GroEL–antibodies (data not presented).

In the present work the recombinant protein was received via expression of *GroEL* gene — bacterial analog of mammal chaperon Hsp–60. That's why it looks reasonable to check immunochemical cross–reactivity of chaperons of different origin — from prokaryotes to mammals. The results of determination of immunoreactivity of polyclonal affinity–purified anti–Hsp–60 antibodies are presented on Fig. 3. It is shown that anti–Hsp–60 antibodies react with polypeptide with molecular weight 60 kDa in the cell lysates of all species (from mice to human) with immunoreactivity comparable to that for the control GroEL antigen. Those data as well as

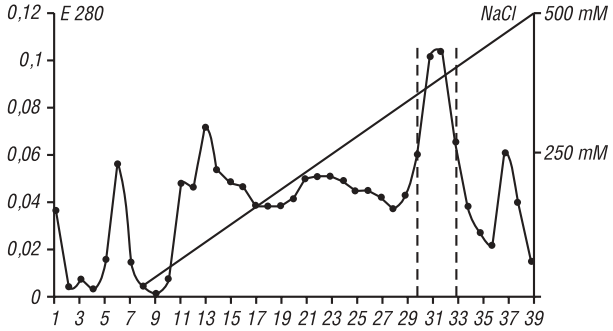

Fig. 1. Ion–exchange chromatography of GroEl (Hsp 60) chaperon on DEAE–Tyopearl 650M column. Fractions 31–34 contain Hsp–60 of 90% purity

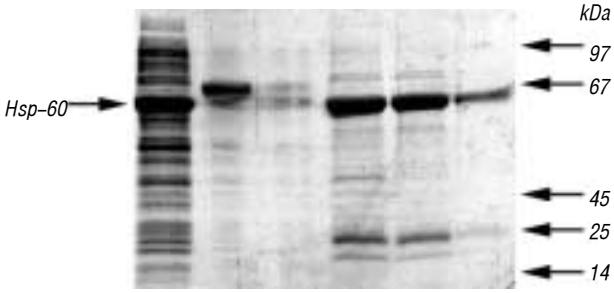

Fig. 2. Electrophoregramm of Hsp 60 chaperon preparation on different stages of purification: 1 — after precipitation with ammonium sulphate; 2, 3 — after gel–filtration on Sephacryl–300 (peak fractions); 4–6 — after final purification on DEAE–Tyopearl 650M column (peak fractions)

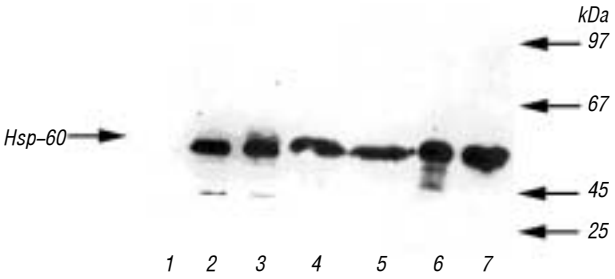

Fig. 3. Western–blot analysis of immunoreactivity of Hsp–60 chaperons in mitochondrial lyzates (2, 4, 6) and cytoplasmic post–mitochondrial supernatants (3, 5, 7), obtained from myocardial cells of different mammals: 1– bovine serum albumin; 2, 3 — myocardium of Balb/c mice; 4, 5 — rabbit myocardium; 6, 7 — human myocardium

data of other authors supporting high homology of Hsp–60 chaperons, allows us to use the recombinant protein as antigen in the research of the role of anti–Hsp–60 autoantibodies in DDB development.

By ELISA with the use of recombinant antigen Hsp–60 we have shown that 80% of the studied sera samples of DDB patients were anti– Hsp–60–positive (Fig. 4). The level of anti–Hsp–60 autoantibodies in the sera of DDB patients was significantly higher ($p < 0.05$) than in the sera of healthy donors. Those data point to the active autoimmune processes in DDB patients and may serve as additional marker of the development of destructive processes in the breast tissues. We have detected also the significant increase ($p < 0.01$) of anti–Hsp–60 antibodies in the blood serum of patients with simultaneous tumor processes (in DDB patients with fibromyoma of uteri, hyperplasia of endometrium and nodular thyroid pathology) (Fig. 4, cases 1, 4, 23–25). However, low number of observed cases is not allowing to made conclusions about the correlation in autoimmune pathology and the risk of breast cancer development.

By immunoblotting technique it was determined that nearly all antibody–positive sera samples contain autoantibodies recognizing Hsp–60 determinants of conformational and sequencial types (data not presented).

The medium level of anti–Hsp–60 immunoreactivity of the sera of DDB patients was notably higher then that for anti–DNA–topoisomerase I. This fact may evidence that the basic alterations in the functioning of breast cells in DDB patients occur on posttranslation stage. This may be caused by alteration of expression

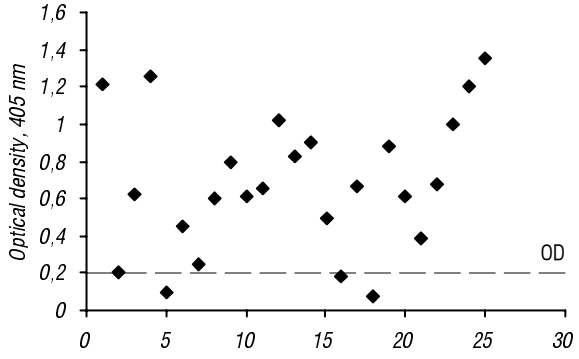

Fig. 4. Sera anti–Hsp 60 reactivity of the patients determined by ELISA. Line 2 OD — medium value of immunoreactivity of the sera of healthy donors (women at postmenopausal age)

and/or activity of chaperons (cytoplasmic as well as mitochondrial or nuclear) resulting in appearance of proteins with wrong folding and disturbances in protein import into organells. From other hand, the involvement of some chaperons in the formation of steroid receptors has been reported [21]; thus, any alterations in activity and expression level of chaperons may affect signal transduction pathways.

The present research demonstrates the first step in the study of the involvement of chaperons in development of tumor processes in DDB patients of perimenopausal age.

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