EXPERIMENTAL STUDY OF HEAT SHOCK PROTEINS IN VACCINOTHERAPY OF MALIGNANCIES

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Summary: Vaccine produced using heat shock proteins (HSP) have significant therapeutic potential in the treatment of malignant neoplasms. One of the ways to increase the effectiveness of autologous HSP vaccines is the modification of tumor-associated antigens by cytotoxic protein-containing metabolites Bacillus subtilis B-7025. This paper discribes the induction and accumulation of heat shock proteins with Mr 70kDa in tumor cells for account of hyperthermia caused by ultrahigh frequency generator and research of the antineoplastic efficacy of vaccines engineered on the basis of tumor cells enriched by heat shock proteins and metabolites of Bacillus subtilis B-7025. These vaccines in terms of growth inhibition of tumor node and antimetastatic effects were found to be more effective than vaccines produced by traditional technology.

Keywords: heat shock proteins, cancer vaccines, protein-containing metabolites, tumor antigens.

Introduction: Vaccine therapy of tumors was formed as original perspective direction of treatment of patients with malignant neoplasias, since adequate immune protection degree in general determines antitumor therapy result.

Antitumor vaccines (AV) are used for development of specific immune response to tumor antigens and they significantly increase effectiveness of oncological patients treatment [1 - 4]. Number of vaccines is available that were constructed based on patients' tumor material, which was modified with different methods. However, during its application the number of obstacles appear: organism's immunologic tolerance to tumor antigens [5], possibility of autoimmune processes induction [6], and rather low altered antigens expression on tumor cells (TC) surface [7].

Among the successful strategies of effectiveness increase of malignant tumors vaccine therapy is combined application of immunotropic substances of natural and synthetic origin in AV [8-10], and among them special position hold heat-shock proteins (HSP). It is known that HSP isolated from TC form complex with wide spectrum of cellular peptides and theoretically can bear on themselves all the antigens peculiar to this specific tumor. Therefore, HSP immunogenicity results from two different peculiarities: peptide-dependent possibility to induce adoptive cytotoxic T-lymphocyte response to antigenic peptides, and peptide-independent immunomodulation activity [11, 12]. Accordingly, application of autologous AV based on HSP complexes with tumor-associated antigens (HSP-peptide complexes) becomes very attractive. However, in spite of successful results of immunotherapy with autologous HSP-vaccines, important problem of insufficient tumor antigens immunogenicity remains.

One of the ways of AV effectiveness increase is intensification of the immune response to tumor-associated antigens by means of their modification with cytotoxic protein-containing metabolites (PCM) of *Bacillus subtilis B-7025.*

Previously it was shown that PCM of *B. subtilis B-7025* increase immunogenicity of that weak oncofetal antigens, like CEA (cancer-embryonic antigen) and AFP (alpha-fetoprotein). This is manifested by significant increase of antibodies titers to such antigens [13]. Effectiveness of vaccines that were prepared from autologous (homologous) TC by its PCM of *B. subtilis B-7025* treatment was proved in numerous experimental and clinical studies [14, 15]. These data formed a basis for construction and further antitumor activity exploration of autologous AV based on HSP complex with tumor antigens that contain cytotoxic *B. subtilis B-7025* PCM as adjuvant.

Aim of the work: to construct antitumor autovaccine enriched in HSP, and to study its therapeutic effectiveness.

Objects and methods of the study. Experiments were conducted on C57B1/6 male mice (age 2 months, body mass 18-20 g) that were received from the breeding house of R. E. Kavetskyi Institute of Experimental Pathology, Oncology and Radiobiology of the NAS of Ukraine, certified in compliance with demands of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes. As tumor growth model Lewis lung carcinoma (LLC) and Ehrlich's cancer (EC) were used. TC was inoculated into animals' thigh in a dose 10^6 cells /animal by standard procedure.

For HSP induction tumor (size up to 1 cm in diameter) heating was provided at temperature 43.0 ± 0.3^0C for 1 hour with experimental generator (frequency 434 MHz, "Istok", Russian Federation). Proteins from tumor tissue (EC, LLC) that underwent local hyperthermia (HT) were prepared by EDTA extraction. Native

proteins separation and assessment of protein content alterations were determined by SDS-electrophoresis (Laemmli's method). To confirm HSP presence and to determine its level in extracts prepared from TC we carried out immunoblot analysis with application of monoclonal antibodies to HSP-70 (company ENZO, USA).

We constructed several AV by using microbial synthesis products of *B. subtilis B-7025* that possess cytotoxic activity TC in the system *in vitro* - PCM with molecular mass 70 and 18.5 kD (PCM70 and PCM18.5), and extracts prepared from tumors (EC and LLC) that underwent HT effect, and also extracts without HT. The vaccines were prepared basing on protein estimation (0.3 mg of TC extracts and 0.3 mg of appropriate *B. subtilis B-7025* PCM by 1 ml of vaccine). Animals' immunization started at the 2-nd day after tumor inoculation, it was provided 4-fold (days 2, 5, 12 and 19), AV dose was 0.2 mg/injection (0.8 mg for complete vaccination course).

The following groups were formed: 1 – tumor growth control; 2 – vaccine based on TC extract (without preliminary HT) and PCM70; 3 – vaccine on the base of TC extract (after HT) and PCM70; 4 - – vaccine on the base of TC extract (without preliminary HT) and PCM18.5 kD; 5 – vaccine on the base of TC extract (after HT) and PCM18.5).

We carried out animals sacrifice and material sampling on day 24 after tumor inoculation, complying with demands established by the European convention for the protection of vertebrate animals used for experimental and other scientific purposes. We assessed antitumor treatment effect by animals' survival

indices, indices of lungs metastases number and volume. We processed experimental study results with generally accepted variation statistics methods with software packages Origin 7.5 and Statistica 6.0 [16].

Results and their discussion: At the first stage, alterations in protein spectrum of extracts prepared from TC that underwent local HT were explored. It was determined that HS expression peak occurred at 24-th hour after heating independently of tumor type. Were received tumor tissue samples (EC and LLC) that underwent local HT effect for the development of optimal technology of effective AV producing from them. By SDS-electrophoresis method, it was shown that there existed difference in protein spectrum between tumor extract that underwent HT effect and those without this effect. It was established that in tumors that underwent local HT proteins concentration with molecular mass about 70, 80, and 10 kD was significantly increased (Figure 1). Especially increased was the concentration of proteins with molecular mass 70 kD, these indices were similar for both tumors.

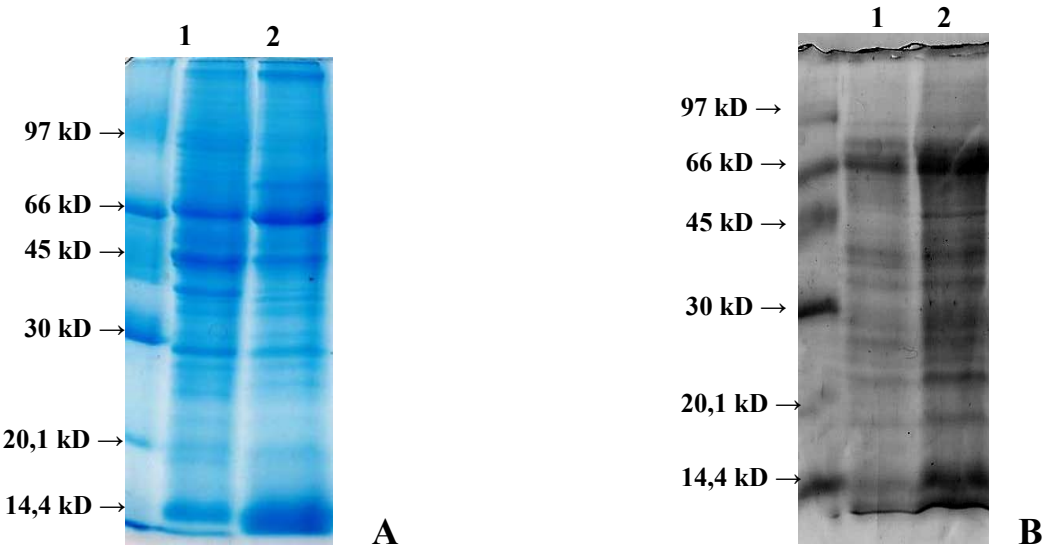

Figure 1. Electrophoregrams of surface proteins of **(A)** Ehrlich's cancer and **(B)** Lewis lung carcinoma (1- before hyperthermia and 2 – after hyperthermia).

Application of *B. subtilis B-7025* PCM (18.5 and 70.0 kD) for AV preparation led to alterations in tumors protein spectrum, presumably, by tumor proteins partial proteolysis (Figure 2).

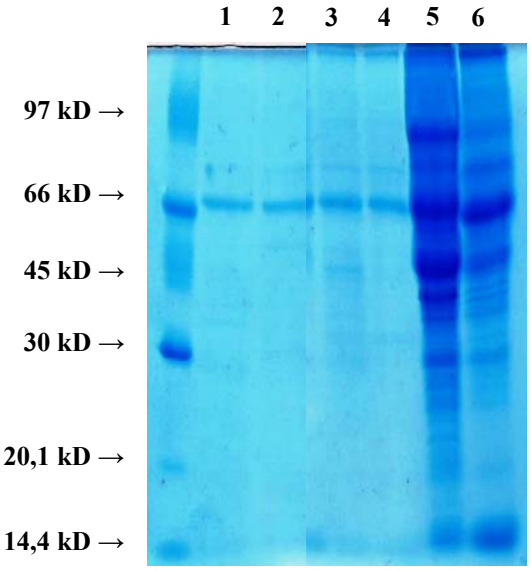

Figure 2. Electrophoretic profiles of vaccines prepared on the basis of Ehrlich's cancer cells (1, 3, 5 – before hyperthermia, 2, 4, 6 – after hyperthermia) and *B. subtilis B-7025* metabolites (1-2 – 70 kD, 3-4 – 18.5 kD).

Besides this, it is known that the component 18.5 kD has proteolytic properties, it refers to class of serine proteases because its action could be blocked by appropriate blocker (PMSF) [17].

Constructed vaccines were studied in experiments *in vivo* in mice with inoculated experimental tumors (EC and LLC). Obtained results demonstrated that

effectiveness of vaccines prepared on the basis of tumors that underwent local HT for HSP enrichment by the number of characteristics exceeded that with traditional technology of vaccines preparation. Better indices of EC growth suppression at its terminal stages (24-th day after tumor inoculation) were observed at administration of the vaccine prepared on the basis of TC that underwent local HT, and *B. subtilis B-7025* PCM with molecular mass 18.5 kD (average tumor volume was 9.35±0.86 cm^3 versus 12.36±1.82 cm^3 in control; index of suppression was 24.35%).

At LLC growth dynamics assessment reliable differences between control and experimental groups were not observed until day 19 of tumor growth ($p>0.05$); on day 22 this difference became evident. In this way in mice that received AV on the base of LLC(HT)+PCM18.5 tumors volume was the smallest and comprised 1.22±0.23 comparing with 1.75±0.19 cm^3 in control animals ($p <0.05$). Primary tumor growth delay was also observed during application of vaccines LLC+PCM18.5; LLC+PCM70 and LLC(HT)+PCM70 (1.48±0.28; 1.31±0.28 and 131±0.24 cm^3, respectively). It should be emphasized that application of TC EDTA-extracts without adjuvants did not have antitumor effect and tumors dimensions were: 2.11±0.34 and 1.80±0.41 cm^3 – at administration of LLC and LLC(HT), that approximated control index..

We assessed LLC metastasing into lungs on 24-th day of the experiment (Table 1).

Table 1

LLC metastising in mice receiving vaccines on the basis of tumor tissue that underwent HT and adjuvants of microbial origin.

Groups of mice	Average metastases number per animal	Average metastases volume per animal (mm^3)
Control	17.17±1.62	28.76±5.09
CLL+PCM18.5	9.20±2.39*	11.40±4.97
CLL(HT)+ PCM18.5	1.57±1.11*	2.02±1.44*
CLL+PCM70	13.67±5.23	19.50±14.05
CLL(HT)+ PCM70	15.29±3.87	17.42±4.63

Pronounced antimetastatic effect was shown with AV, prepared with application of *B. subtilis B-7025* PCM with molecular mass 18.5; at that exactly the vaccine on the basis of extract from LLC cells that underwent hyperthermia (LLC(HT)+PCM18.5) appeared to be the most effective. Average metastases number per animal was only 1.57±1.11 comparing with 17.17±1.62 in control animals, and their volume — 2.02±1.44 and 28.76±5.09, respectively ($p < 0.05$). Antimetastatic effect of mentioned AV significantly exceeded that of vaccines, which were prepared from tumor without preliminary HT. This allows suggesting HSP enrichment of tumor tissue treated with local HT.

As the most frequently used AV are HSP70 [18, 19], therefore for elucidation of mechanisms of possible antitumor effect of the developed vaccine we performed immunoblot analysis of HSP-70 level (with application of monoclonal antibodies to HSP-70 (company ENZO, USA), both in EDTA-extracts prepared from TC that underwent HT, without HT treatment, and also in vaccines prepared with its application (Figure 3).

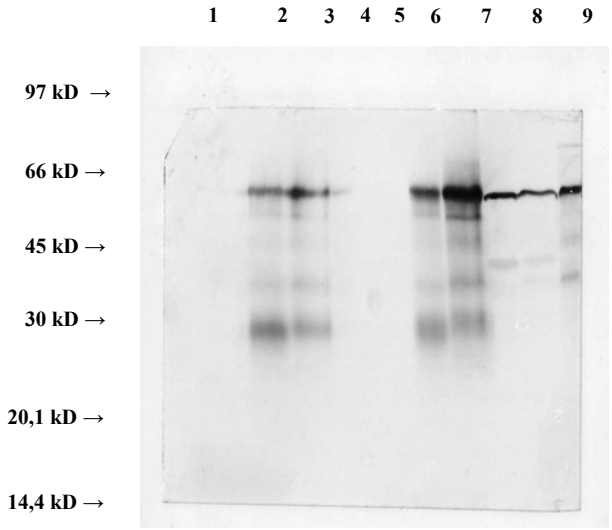

Figure 3. Immunoblot analysis of HSP-70 levels in tumor extracts and vaccines prepared on its basis: 1 —*B. subtilis B-7025* PCM-70 kD; 2 — vaccine LLC + PCM70; 3 — vaccine LLC(HT) + PCM70; 4 — vaccine LLC + PCM18.5; 5 — vaccine LLC(HT) + PCM185; 6 — EDTA-extract of LLC; 7 —EDTA-extract of LLC(HT); 8 —EDTA-extract of EC(HT); 9 — EDTA-extract of EC.

At analysis of HSP-70 level in EDTA-extracts prepared from LLC cells it was demonstrated that tumor HT led to significant increase of HSP-70 level. Addition of PCM of *B. subtilis B-7025* with molecular mass 18.5 and 70 kD to

tumor EDTA-extracts led to reduction of HSP-70 level because under PCM effect its disintegration into constituents with molecular mass about 28 kD occurs.

At immunoblot analysis of HSP level in serum of animals sampled in 24 hours after tumors' heating the difference in HSP-70 amount (comparing with control group) was not determined. Obtained results above all demonstrate that on the second day after tumors' HT increase of HSP-70 level in tumor occurs, but it is not observed in serum of animals - bearers of this tumor. Either this is most likely associated with vessels coagulation in tumor under hyperthermia effect, or maximal HSP-70 synthesis in peripheral blood is observed at later stages.

For confirmation of the obtained results study of HSP-70 level in EDTA-extracts of LLC tumors that underwent local hyperthermia, and vaccines prepared on its base using PCM of *B. subtilis B-7025* with molecular mass.70 and 18.5kD was provided (Figure 4).

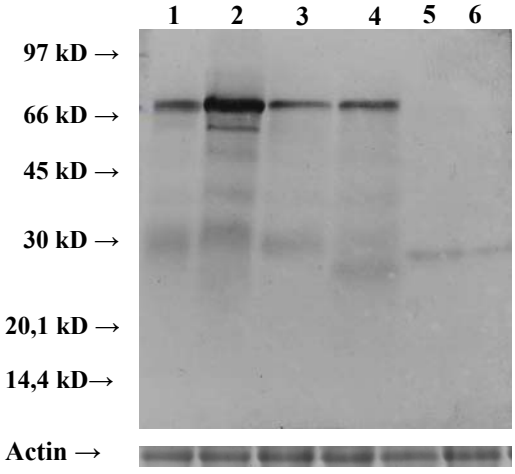

Figure 4. Immunoblot analysis of HSP-70 levels in tumor extracts (1— LLC, 2 — LLC(HT) and vaccines, that were prepared on its basis with application of *B.*

subtilis B-7025 PCM with molecular mass 70 kD (3— LLC+PCM70 and 4 — LLC(HT)+PCM70), or *B. subtilis B-7025* PCM with molecular mass 18.5 kD (5— LLC+PCM18.5 and 6 — LLC(HT)+18.5).

At analysis of immunoblot test results it was confirmed that tumor HT led to increase of HSP-70 level in tumor LLC EDTA-extract. At that PCM 70 kD of *B.subtilis B-7025* in ready vaccine leads to HSP-70 level reduction, and application of *B. subtilis B-7025* PCM 18.5 kD promotes HSP-70 disintegration into constituents with molecular mass about 28 kD that most probably increases its immunogenicity and leads to more pronounced antitumor effect.

In serum of control group animals and animals, which received LLC EDTA-extracts (that underwent local HT and without thermal treatment), HSP-70 level did not differ notably. However, in mice, which were administered with vaccine on the basis of EDTA-extract from LLC tumors that underwent local HT and PCM 18.5 of *B. subtilis B-7025,* in which pronounced antimetastatic effect was registered, HSP-70 in blood serum was practically absent. From this preliminary assumption can be made that HSP-70 level in blood serum may reduce at effective antitumor therapy.

CONCLUSIONS

In that way, the results of HSP expression examination in model tumors under local HT conditions indicate considerable increase of HSP70 level in them. Previous attempts of AV preparation on the basis of tumor tissue EDTA-extracts enriched with HSP70 by adjuvants of microbial origin have positive results that

are expressed in better antitumor and antimetastatic effectiveness of these vaccines comparing with vaccines prepared according to standard technology. Decrease of HSP70 level in blood serum of vaccinated mice correlates with study vaccines antitumor effect. This data indicate necessity of further detailed development of all the stages of AV preparation that should allow preserving the benefits of HSP enrichment and provide with maximal vaccination effect.

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