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COMMENTARY

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HSP70-based anti-cancer immunotherapy

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ABSTRACT

Heat shock protein 70, (Hsp70) constitutes a powerful system of cytoprotection in all organisms studied to date. Exerting such activity, Hsp70 rescues cancer cells from antitumor therapy, posing a great challenge for oncologists. In contrast to its protective action, Hsp70 was found to be released from cancer cells, prompting cytotoxic lymphocytes to target and kill the tumor. A great number of vaccines have been developed on the basis of the ability of Hsp70 to present tumor antigen or to elevate the sensitivity of cancer cells to cytotoxic lymphocytes. In this commentary, we consider novel data on the employment of pure Hsp70 in the therapy of glioma and melanoma malignancies. We show that intratumorally delivered Hsp70 penetrates cancer cells and pulls its intracellular analog outside of the cell. This displacement may activate cells, constituting both innate and adaptive immunity. *In vivo* delivery of Hsp70 was found to inhibit tumor growth and to extend survival. The technology of intratumoral injection of pure Hsp70 passed through preclinical trials and was investigated in clinics for children with brain cancer; the results show the safety and feasibility of a new approach.

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Immunotolerance of tumors and heat shock response

Malignant tumors evade immune attack of the host, although cancer cells themselves have all features necessary for the activation of host immune response: they often bear mutated or aberrant proteins, which can trigger the immune reaction of the body by displaying tumor-associated antigens (TAA) and by stimulation of inflammation at the tumor site.[1] According to a well-accepted opinion, immune-suppressive mechanisms are provided by cells inhabiting the tumor microenvironment, including T-cells, macrophages, dendritic cells (DC), myeloid derived suppressive cells (MDSC) and some other representatives of the immune system. Together with tumors, these cells supply the microenvironment with immunosuppressive cytokines, such as IL-10 and TGF-β, which leads to a concentration of MDSC at the tumor location, activation of FoxP3+ regulatory T-cells (Tregs), polarization of tumor-associated macrophages to the M2 phenotype and inhibition of T-cell proliferation.[2] The paradox is that, instead of protecting the organism against hyperplasia, the above cells defend the tumor by constantly making the latter more aggressive, actively metastasizing.

To overcome the above-mentioned tolerance, immunotherapeutic technologies based on strong adjuvants and powerful triggers of both innate and adaptive antitumor immunity are warranted. Molecular chaperones or heat shock proteins exert both activities in numerous simulations and vaccine constructs. The heat-shock response is the universal system of cellular reaction to a variety of stressful or toxic factors; the reaction is triggered by a special transcription factor (HSF1) and causes the synthesis of a large group of heat shock proteins belonging to different families commonly designated by the molecular mass of a typical representative, *e.g.*, Hsp27, Hsp40, Hsp70, Hsp90, Hsp110, and some others. Most Hsps were found to be molecular chaperones that formed a system of protein quality control and participated in almost all functions of a cell. It is important that the heat shock response triggered by HSF1 is most effective in stem-like cancer cells; in these cells, it controls the expression of hundreds of genes necessary for cell survival in extremely toxic surroundings or in conditions of an anticancer therapy.[3] Moreover, stroma cells of the tumor microenvironment demonstrate strong activation of Hsf1, a process induced by highly tumorigenic cells and indicating that the heat-shock response is a significant factor of reprogramming the cells inhabiting a tumor niche.[4] Many Hsps possess cytoprotective activity and their enhanced expression typical of cancer cells poses a substantial problem for anticancer therapy (for review see ref. 5). Fortunately, this obstacle can be circumvented by employing the specific properties of different Hsps in the design of anticancer vaccines.

Heat shock proteins in anti-cancer vaccines

One of the most frequently used in Hsp-based vaccine proteins is Grp96 (glucose-regulated protein), which forms tight complexes with TAA from colorectal cancer, pancreatic adenocarcinoma and melanoma.[6-8] Other candidates for chaperonic vaccine are the high-molecular weight heat shock proteins, Hsp110 and Hsp170

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(Grp170). These two chaperones were shown to be effective when combined in an immunization complex with known tumor antigens, gp100 and intracellular domain of human epidermal growth factor-2 (HER-2). The appropriate fusion constructs suppressed B16 melanoma growth[9] and mammary tumor development in FBV-neo transgenic mice.[10] Another Hsp, Hsp60, was also shown to exert immunomodulatory capacity when its gene was fused into a DNA vaccine construct with papilloma virus tumor antigens.[11] Interestingly, the mixture of Hsps purified from mouse sarcoma containing Hsp60, Hsp70, Grp96, and Hsp110, also demonstrated a profound anticancer immune response in autologous tumors.[12]

In this Commentary, we pay special attention to Hsp70-based vaccines. The Hsp70 family of closely related genes encodes 11 to 14 proteins in mammalian cells, of which 2 may be induced by a great number of harmful and/or therapeutic factors. Elevated synthesis of Hsp70 causes more efficient function of the chaperonic machinery and leads to a reduction of cell sensitivity to repeated action of the same or of other stressful agents, *e.g.*, to the development of a tolerant state. The majority of cancer cells explored to date contain a high amount of Hsp70 and therefore, are protected from environmental stressors; most typical of the latter is oxidative stress, whose deleterious effect was found to depend on the chaperone content.[13]

Thus, Hsp70 as do many other molecular chaperones, plays a dual role in cancer cells: it protects the latter from natural stressors and antitumor drugs and, when transported to the exterior of a cell, it can represent its antigenic structures to natural killer cells or to dendritic cells. Activation of both types of immune systems by Hsp70 was convincingly proven in studies by several groups (for review see ref. 14). P. Srivastava was the first to discover the development of the individual Hsp70-mediated response in animals immunized with preparations of their own chaperone.[15] In these works, Hsp70 was isolated from the malignant tissue using the protocol omitting the step of chromatography on adenosine-triphosphate-agarose. The release of the TAA bound to ATP made the preparation immunologically ineffective, thus suggesting the importance of keeping the intact complex Hsp70-TAA. These studies provoked a great number of anticancer vaccines based on the property of Hsp70 to bind TAA and to process these in APC; the samples of individual cancers were used to immunize the patients.[16]

The technology of personal immunotherapy was subjected to clinical trials to cure high-risk breast cancer and chronic myelogenous leukemia from 2001 to 2008, and though the results were not perfect (www.clinicaltrials.gov), the development of more efficient vaccine constructions continues to date, focusing on newer methodological aspects (see Table 1). First, instead of Hsp70, another stress protein, Grp96 purified from cancerous tissue, was used. The latter vaccine, named Oncophage, was also subjected to clinical trials in which the protein alone or coupled with Gleevec™ was administered to oncological patients (Novartis, Agenus, Inc. 2012). Secondly, in many settings, Hsp70 coupled to a certain peptide antigen derived, for instance, from papilloma virus (EP7) was employed to immunize the animals in a therapeutic setting; the efficacy of such vaccines strongly depends on a tumor origin and antigen presented by the chaperone (for a review see ref. 27). One of the recent reports describes the application of mesothelin in complex with mycobacterial Hsp70 in a mouse model of ovarian cancer and mesothelioma.[28] Particularly high anticancer activity was found when the animals were immunized with the Hsp70-TAA complex isolated from fusions of dendritic and tumor cells; this immunization resulted in a T cell-mediated immune response including a significant increase in CD8+ T cells and induction of effector and memory T cells.[26] Thirdly, DNA vaccines can be employed instead of protein-peptide complexes. In these constructs, the Hsp70 gene is fused with a certain sequence of known TAA and expected to be produced in a host tissue (see Table 1). Another group of immunotherapeutic tools is based on the discovery of the surface-bound form of Hsp70; this part of Hsp70 molecules appears on the outer membrane of cancer cells treated with certain factors, one of which was shown to be mild heat shock.[29] The membrane-bound Hsp70 exposes its 14-mer TKD peptide at the cell surface, which serves also as an effective transport-promoting domain for the whole Hsp70 molecule.[30] One of the important features of the TKD peptide is its specific recognition by cytotoxic NK cells and several technologies were developed employing this kind of tumor cell recognition and eradication (see Table 1). Among these approaches are administration of certain anticancer drugs, photodynamic therapy, X-ray therapy, and others.[31,32] Because most cancer cells express TKD peptide on their surface, even under normal conditions, the claim was made that the peptide was a potent tumorigenicity marker, and specific antibody to the peptide was generated and employed in therapeutic modalities.[32] Because most Hsp70-based vaccines use the activity of extracellular chaperone or of its domain exposed on a cell surface, it seemed worthwhile to test whether pure Hsp70 delivered to the tumor might induce an anti-cancer immune response. In one such study, recombinant Hsp70 was delivered inside B16 mouse melanoma, causing a delay in tumor growth. The therapeutic effect was enhanced if the protein was injected together with magnetic nanoparticles to heat the tumor and this combinative therapy

Table 1. Immunomodulatory activities of Hsp70.

Form of Hsp70 or vaccine construct	Immune effect	Reference
Immunization with Hsp70/Grp96 isolated from individual tumor response (Oncophage)	Personalized CD8+ cell-mediated anti-tumor response	17,18
Exposition of Hsp70 on surface of cancer cells treated by ionizing radiation PDT	Elevated NK cell toxicity	19
Exposition of Hsp70 on surface of cancer cells subjected to photodynamic therapy	DC maturation and T cell activation	20
Immunization with a complex Hsp70-HPV16 oE7 antigen	CD8+ cell mediated response.	21
Gene therapy with adenovirus vector carrying the Hsp70 gene (AdCEAp-Hsp70)	CD4+/CD8+ cell-mediated response; gamma-delta T cell activation	22
Exosomes from Hsp70-overexpressing tumor cells	DC maturation, CD4+/CD8+-mediated response	23
Immunization with complex Hsp70-alpha-fetoprotein	CD8+ cell-mediated cytotoxicity	24
Ectopic expression of Hsp70 in cancer cells	CD8+ cell response elevation.	25
Hsp70 isolated from DC-tumor cell fusion product	Full-scale anti-tumor response	26

led to a strong inhibition of tumor growth that was presumably due to an elevation of the specific immune response as follows from the data on interferon-gamma production.[33]

Anti-tumor activity of pure recombinant Hsp70: possible mechanism and therapeutic application

The formulation of anti-cancer vaccine offered by Srivastava in 1990 has a decisive advantage as a personalized approach. However, the method has at least 2 major disadvantages: firstly, the difficulty in obtaining sufficient tumor material for Hsp70-TAA complex isolation and secondly, the high cost of the latter procedure. These problems made the results of phase III clinical trials of personalized Hsp-based vaccine for patients with melanoma upsetting.[34]

In our experiments demonstrating high metastatic activity with rat RA-2 rhabdomyosarcoma,[35,36] we immunized the animals with a preparation of Hsp70 purified from bovine muscle and the latter mixture with tumor extract; a second preparation was administered because it might give a more pronounced immune response to TAA of RA-2. Earlier, this mixture was found to give a profound antitumor response when employed as a therapeutic modality. The unexpected result was that the most efficient reduction in tumor lesion number and size was

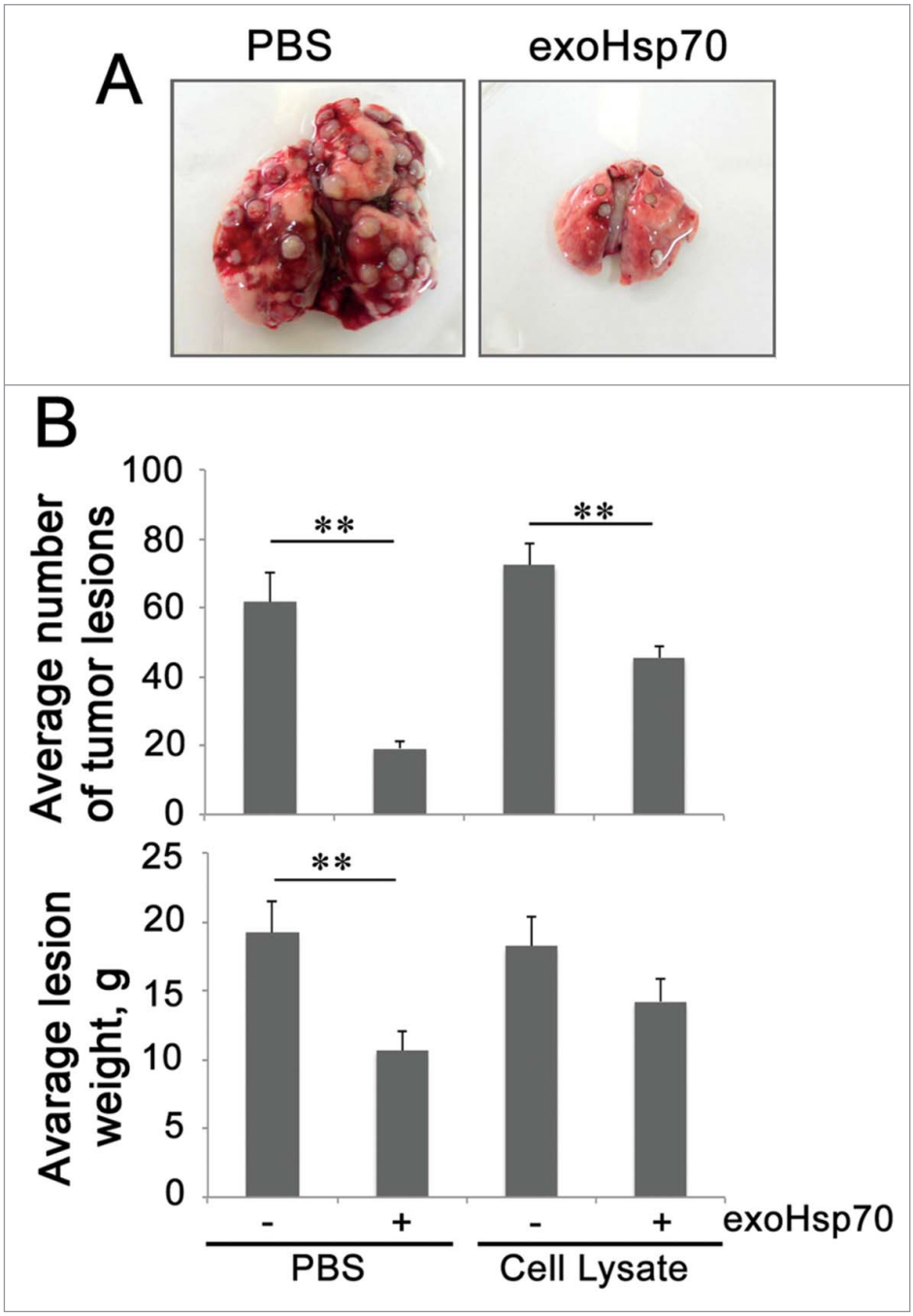

Figure 1. Administration of pure Hsp70 demonstrates a more profound anti-tumor prophylactic effect in a model of RA-2 rat rhabdomyosarcoma as compared with an Hsp70 mixture with tumor antigens. A. Lungs of experimental animals preliminary injected i.v. with PBS or Hsp70 alone or mixed with tumor extract were excised on the 21st day after tumor cell administration. B. Average number and weight of tumor lesions in experimental groups.

found in rats injected with pure Hsp70[37] (Fig. 1). These data prompted us to study in more detail the immunomodulatory effect of pure Hsp70 delivered into tumors of other origins. Pure human recombinant Hsp70 was injected singly or via Alzet micropump into brains of rats with intracranial C6 glioma tumor. Such injections, particularly those done using an osmotic pump, caused a significant delay in tumor growth and increase the survival of tumor-bearing animals. Importantly, the therapeutic effect was accompanied by the growth of specific CD4+ and CD8+ T lymphocytes and activation of cytotoxic NK cells.[38] In the other setting, Hsp70 in a hydrogel composition was applied on the skin surface on top of B16 mouse melanoma. In this case, we also observed substantial inhibition of tumor growth as well as an elevation of the survival rate.[39,40]

To study the mechanism of Hsp70 immunomodulatory effects, *in vitro* experiments were carried out in which C6 glioma or B16 melanoma cells incubated with fluorescently labeled Hsp70 were stained with antibody specifically recognizing the TKD-peptide of Hsp70. To our surprise, we

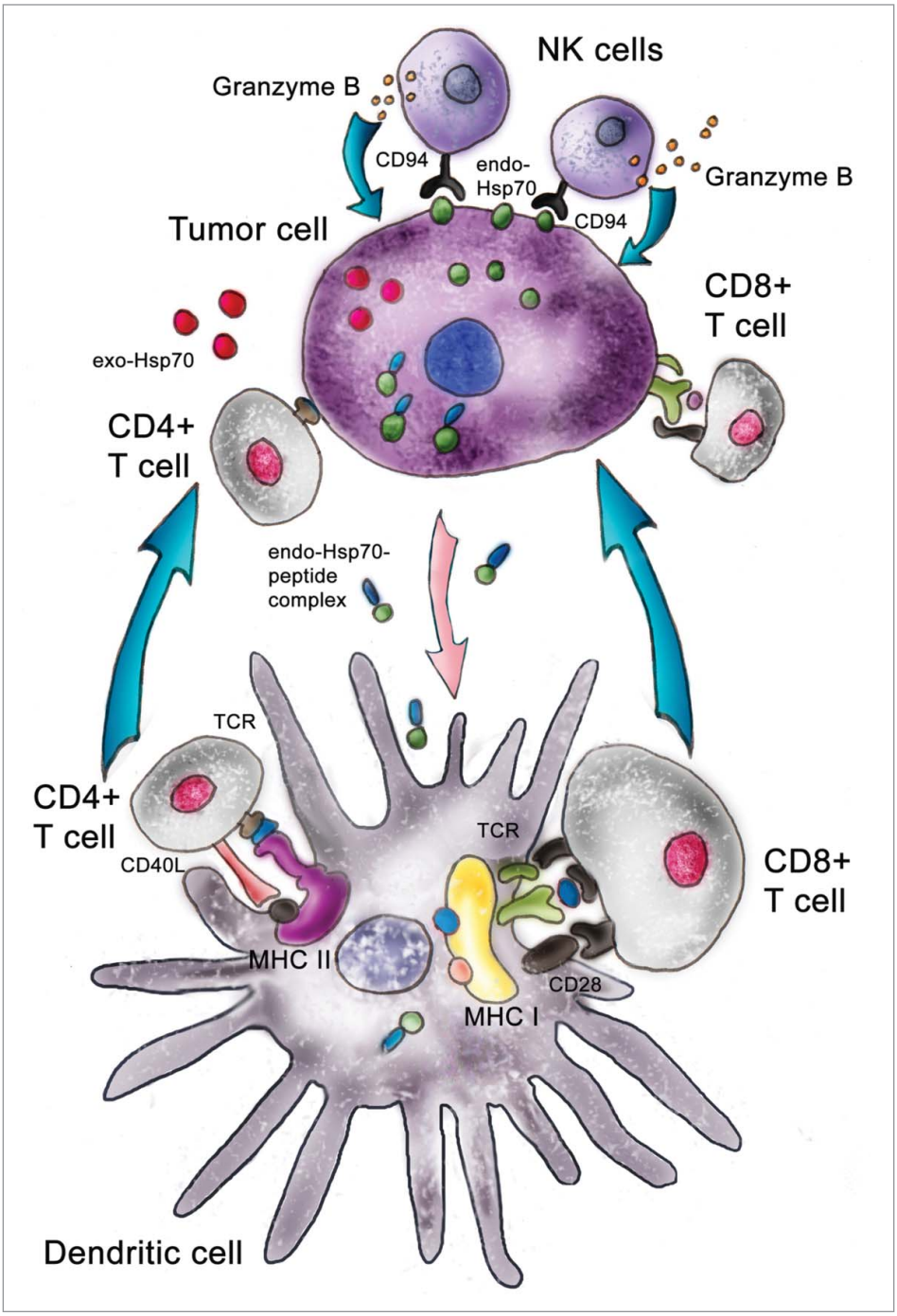

Figure 2. Pathways used by intratumorally delivered Hsp70. Pure Hsp70 penetrates inside a tumor cell and withdraws its intracellular analog to the outer membrane; this surface-attached Hsp70 is targeted by cytotoxic lymphocytes, NK cells. Exogenous Hsp70 occurring inside a tumor cell pulls out its endogenous counterpart, which transfers tumor antigens to dendritic cells, which present these in complex with MHC class I or class II antigens. Using different receptor structures, mature DCs activate CD8+ and CD4+ lymphocytes and trigger this specific cytotoxic effect.

observed that only formerly endogenous Hsp70 was presented on the cancer cell surface while exogenous chaperone passed through the cell body and was released without delay on the plasma membrane. The most interesting observation was that exogenous Hsp70, by extrusion of its cellular analog, increased the sensitivity of tumor cells to cytotoxic lymphocytes in the appropriate assay.[41] Using affinity chromatography, we found that besides the effect of displacement of its endogenous counterpart, exogenous Hsp70 causes the former to be released into the extracellular milieu. Thus, the hypothetical mechanism of pure Hsp70 added to a cancer cell culture or injected intratumorally may function along 2 pathways. First, exposure of Hsp70 on the exterior side of the plasma membrane makes cancer cells accessible to cytotoxic lymphocytes, NK cells (Fig. 2, upper part). This recognition may be performed by CD94 receptors of NK cells and leads to a release of Granzyme B molecules that attack a target tumor cell. This view completely agrees with data from the Multhoff lab.[42] Another pathway is activated by the efflux of Hsp70 molecules, presumably carrying TAA from cells affected by exogenous Hsp70; this flow can also be a result of tumor cell disruption due to the attack of cytotoxic cells (Fig. 2, lower part). According to widely spread opinion, Hsp70 released from tumor cells penetrates inside DC where TAA can be presented in context with MHC Class I or Class II antigen complexes. MHC class II receptor-mediated complexes bind to T-cell receptors on CD4+ cells, whereas MHC class I interacts with CD8+ cells, giving rise to the expansion of the cytotoxic cell population.[43] Both components of the general anticancer immune response, innate and adaptive, are presented in the technology of intratumoral delivery of pure Hsp70. This technology was recently passed through preclinical trials and was subjected to limited investigation in the Children's Brain Cancer Clinics of the Polenov's Russian Research Institute of Neurosurgery in St. Petersburg. The study demonstrated the safety of recombinant Hsp70 and feasibility of its intratumoral delivery in patients with brain cancers.[44] The experiments were initiated in 2011 and the follow-up period was 12 months; in 2012, experimental clinical investigations were stopped in Russia according to new Federal law. The information as of March, 2016 shows that 11 of the 12 patients who received intratumoral injections of the chaperone are alive, and this is the best argument in favor of Hsp70-based anti-tumor therapy.

Abbreviations

DC	dendritic cells
HSF1	heat shock factor
Hsp	heat shock protein
Grp	glucose regulated protein
HER-2	human epidermal growth factor-2
IL-10	interleukin-10
MDSC	myeloid derived suppressive cells
MHC	major histocompatibility complex
NK cell	natural killer cells
TAA	tumor-associated antigens
TGF-β	tumor growth factor–β
Tregs	regulatory T-cells

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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