Minireview

Overcoming Immune Tolerance to Cancer by Heat Shock Protein Vaccines[1]

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Abstract

Heat shock proteins (HSPs) are a large family of inducible but also ubiquitously and constitutively expressed protein chaperones involved in assisting protein folding and unfolding in the cells. The realization of the immunological significance of HSPs came from the observation that tumor cell-derived HSPs could immunize against tumors, although there were no structural differences between HSPs from normal cells and from cancer cells. Studies of this puzzle have uncovered two unique immunological properties of HSPs. One is their ability to associate and present antigenic fingerprints/peptides of cells to MHC antigens. The other is their ability to activate dendritic cells (DCs), which are the most efficient antigen-presenting cells. These surprising immunological attributes of HSPs are now the basis for a number of already completed clinical trials for cancer immunotherapy. Because DCs orchestrate both innate and adaptive immunity, the findings that HSPs can modulate the functions of DCs may have fundamental implications. We hypothesize that HSPs are at the forefront in counterbalancing T-cell tolerance to tumor-associated antigens and thus play pivotal roles in antitumor immunity *in vivo*.

Introduction

HSPs[3] are also referred to in the literature as stress proteins or protein chaperones, although most HSPs are ubiquitously and constitutively expressed. They are a family of proteins that play fundamental roles in all cellular events involving protein folding and unfolding (1–3).

The connection of HSP with tumor immunity was discovered serendipitously in the 1980s (4–6). It was found that structurally unaltered HSPs, purified from tumor cells, could immunize animals to generate tumor-specific immunity, whereas corresponding preparations from normal tissues did not. The earliest studies were carried out with an endoplasmic reticular HSP, gp96 (7, 8), but similar results were later obtained with hsp70 (9), hsp90 (10, 11), calreticulin (12, 13), hsp110, and grp170 (14). The immunogenicity of tumor-derived HSPs was shown to be dependent on the peptides associated with HSP molecules but not against HSPs *per se*. Not surprisingly, HSPs isolated from cells infected with viruses or bacteria could be used to immunize against the respective viruses (15–18), or intracellular organisms (19, 20), because of the ability of HSPs to chaperone corresponding antigens.

The ability of HSPs to potentially bind to the whole cellular peptide repertoire makes them attractive candidates for cancer vaccines. The advantages are 2-fold: (*a*) cancer vaccines, based on HSP-peptide complexes, entirely bypass the need for knowing the identity of tumor-specific peptides; and (*b*) this approach delivers multivalent antigens for potential multifarious immune responses.

The practical implications of HSPs are now being examined by a number of well-designed clinical trials. HSPs may play yet more fundamental roles in immune responses because of a series of surprising findings that they can modulate the functions of DCs in a receptor-dependent manner.

HSPs as the "Swiss Army Knives of Immunology"

The coordinated response by the innate immunity and the adaptive immunity is essential for efficient immune response. Taking antitumor immunity as an example, the first defense could well be innate immunity mediated by NK cells (21, 22). Then NK-mediated cell lysis of tumor cells can conceivably lead to cross-presentation of antigens by DCs to prime adaptive T-cell immunity. Activated T cells can produce cytokines or express CD40 ligand on their cell surface to reciprocally activate DCs.

Increasing evidence suggests that HSPs may play important roles in both innate and adaptive immunity. In combating against infections, DCs can be activated by a large number of microbial molecules, such as lipopolysaccharide, and unmethylated CpGs, which then trigger adaptive T- and B-cell immunity (23). For generating immune responses against tumors, allografts, and other self antigens, there is a need for endogenous nonmicrobial molecules that activate DCs (24). Studies thus far suggest that HSPs could be such endogenous molecules that activate DCs (25). The initial clues were derived largely from the analysis of the responses of the immune system to a purified endoplasmic reticular HSP, gp96 (26). It was found that the interaction of purified gp96-peptide complexes with APCs, such as macrophages or DCs, leads to the binding of gp96 to the common HSP receptor, CD91, on APCs (27, 28), followed by internalization of the gp96-peptide complexes, processing of the gp96-

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[3] The abbreviations used are: HSP, heat shock protein; DC, dendritic cell; NK, natural killer; APC, antigen-presenting cell; CR, complete response.

chaperoned peptides, and their re-presentation by MHC I and MHC II molecules. The MHC peptide complexes now function as "signal one" to stimulate the cognate $CD4^+$ and $CD8^+$ T cells. Moreover, the interaction of gp96 with APCs also leads to the activation and maturation of DCs, which secrets pro-inflammatory cytokines and provides costimulatory signals ("signal two") for effective T-cell priming. These findings have been independently verified by using cells expressing gp96 on the cell surface (29). It was found that cell surface expression of gp96 by tumor cells leads to DC maturation and cross priming of tumor-specific T cells.

The abilities of HSPs to chaperone peptides and to activate DCs seem to be the common features associated with all major HSPs examined thus far including GRP170, HSP110, gp96, HSP90, HSP70, and calreticulin. Their immunological features can thus be summarized as follows: (*a*) HSPs chaperone immunologically important molecules such as MHC I (30), immunoglobulins (31), T-cell receptors, and Toll-like receptors (32); (*b*) HSPs chaperone and bind cellular peptides; (*c*) extracellular HSPs serve as cytokines to activate the innate functions of APCs, such as DCs, because of their binding to specific receptors on APCs; (*d*) HSPs can deliver their chaperoned peptides from non-APCs to MHC molecules of APCs; and (*e*) depending upon different modes of tissue damage, the release of HSPs may play immunoregulatory roles *in vivo*. Thus, they were hailed by some as the immune system's Swiss army knives (33).

HSPs in Clinical Development

Tumor-derived HSPs have been shown to be effective cancer vaccines not only for prophylaxis against cancers but also for the treatment of existing cancers in many preclinical tumor models (4, 26). These have prompted systemic clinical testing of tumor-derived HSPs for the treatment of human malignancy (34, 35). The current effort has been focused mainly on gp96 and HSP70. Unlike traditional cancer drugs, HSP-peptide vaccine is individually based and tailored toward an individual tumor of an individual patient (34). This is based on two reasonings: (*a*) HSPs chaperone antigenic fingerprint of cells from which they are isolated; and (*b*) tumor-protective antigens are individually distinct (or private; Ref. 36). As early as in the 1940s, it was appreciated that tumors were antigenically distinct from one another, most likely because of the subsequent realization of the differences in peptide pools among different tumors, as a result of random mutations of DNAs in the transformed cell. Depending on the tumor types, grade, differentiation stage, or genetic background, peptide pools that are associated with HSPs should also be individually unique. Therefore, to generate effective tumor vaccine for one patient, tumor antigens (in this case, HSP-peptide complexes) have to be derived from autologous tumors of this patient but not from tumors that were obtained from another patient. More than 300 patients have been treated with HSP vaccines thus far (34). The diseases include lymphoma, renal cell carcinoma, melanoma, colorectal cancer, gastric cancer, pancreatic cancer, breast cancer, and others.

Results from three trials have been published fully. Others appeared only in the abstract form, which will not be discussed here. One Phase III trial for the treatment of stage III renal cell carcinoma in the adjuvant setting is ongoing. In the pilot study, 16 patients with a variety of refractory metastatic diseases were immunized s.c. with 25 μg of autologous tumor-derived gp96 once a week for 4 weeks (37). Patients were monitored clinically and immunologically. No significant toxicities including the generation of autoantibodies were reported. Subsequent studies confirmed the conclusion that there were no acceptable side effects or autoimmune phenomenon associated with gp96 vaccination. Six of 12 patients had increased MHC class I-restricted IFN-γ-producing $CD8^+$ T cells that were specific against autologous tumors or tumor membranes, as measured by a quantitative ELISPOT assay. Eight of 13 patients had expanded NK cell populations in the peripheral blood.

The ability of human melanoma-derived HSP70 to stimulate autologous melanoma-specific T cells for producing IFN-γ were demonstrated by using peripheral monocytes pulsed with HSP70 as targets (37). This stimulation was MHC class I dependent and antigen specific. These data have prompted a larger Phase II trial testing the HSP70 for the treatment of advanced melanoma (38). Sixty-four eligible patients ($n = 64$) were surgically resected of metastatic tissue to obtain tumors required for vaccine production. Forty-two patients were able to receive the vaccine, and 39 were evaluable after one cycle of vaccination (four weekly injections).

No treatment-related grade IV toxicities were observed. Of the 28 patients with measurable disease, 2 had a CR and 3 had stable disease at the end of follow-up. Durations of CRs were 559+ and 703+ days, whereas stable disease durations lasted for 153, 191, and 272 days, respectively. Three of the 11 patients rendered disease free by surgery experienced a long-term disease-free survival of 25231, 36645, and 6421+ days. ELISPOT assay with peripheral blood mononuclear cells of 23 subjects showed a significantly increased number of postvaccination, melanoma-specific, T-cell spots in 11 patients, with clinical responders displaying a high frequency of increased T-cell activity. Immunohistochemical staining of melanoma tissues from which vaccine was produced revealed high expression of both HLA class I and melanoma antigens in 7 of 8 clinical responders (12 CR, 3 stable disease, and 3 long-term disease-free survival) but in 4 of 12 nonresponders. There is currently a multi-institute worldwide randomized Phase III study of autologous tumor-derived gp96 vaccination for patients with stage III renal cell cancer after curative surgery. Relapse-free survival and overall survival will be the sole objectives of this study. This study is actively enrolling patients.

HSPs in Breaking Immunological Tolerance: A Hypothesis

One of the key features of the immune system is its ability to mount effective immune responses against pathogens while remaining nonresponsive to more abundant and normal self-antigens. For T lymphocytes, the vast majority of potentially self-reactive cells are eliminated during development in the thymus, a process called negative selection (39, 40). When self-reactive T cells do migrate into the periphery, multiple

mechanisms are in place to prevent these cells from inappropriate activation, including antigen sequestration, clonal exhaustion, anergy, and antigen-specific suppression or regulation (41). The prevalent hypothesis regarding the mechanisms for antigen-driven peripheral tolerance is that antigen alone (signal 1), without the presence of costimulatory molecules (signal 2), can lead to antigen-specific unresponsiveness or anergy (24, 42, 43). The de fault pathway for immunological response to tumor thus might be tolerance attributable to lack of signal 2 (24). This tolerance can generally be overcome by manipulating signal 2, such as transfecting of tumor cells with costimulatory molecules (44, 45), introducing proinflammatory cytokines/chemokines to the tumors (46, 47), administration of systemic cytokines or signal 2 agonist (48, 49), and other means to activate/modulate the function of DCs (50, 51). Because HSPs can activate DCs to up-regulate signal 2, in addition to delivering signal 1 through cross-presentation of HSP-chaperoned peptides, we hypothesize that HSPs can break peripheral tolerance against tumor associated antigens. Our hypothesis is supported by a number of observations: (*a*) even in tumor-bearing host when tumor-specific tolerance is presumably induced, immunization with HSPs, such as gp96 and HSP70, can elicit tumor protection in a T-cell-dependent manner (52); (*b*) the priming of tumor-protective $CD8^+$ T-cell-mediated tumor immunity by HSPs can be $CD4^+$ T-cell independent (53–55), highlighting the abilities of HSPs to serve as both peptide chaperones and adjuvants; (*c*) overexpression of HSPs such as HSP70 can lead to increased immunogenicity of poorly immunogenic tumor cells (56, 57); (*d*) it has been shown recently that apoptotic cell death leads to tolerance induction, whereas necrotic cell death is associated with immunity (58, 59). It was proposed and later shown that HSPs are released after necrotic cell death, but not after apoptosis, and hence are likely the major contributors to the immunogenic activities (60–62); (*e*) the extracellular trafficking (either surface expression or secretion) of HSPs such as gp96 (7, 8, 63–67), calreticulin (68, 69), HSP70 (70, 71), and HSP90 (72) has been reported. Both cell surface expression and secretion of HSP gp96 by tumor cells result in increased immunogenicity of the tumor cells; and (*f*) epidemiological studies have suggested the association of increased HSPs with the development of autoimmune diseases. A significant subset of patients with systemic lupus erythematosus, for example, has increased expression of HSP90 (73).

Thus, under the condition of stress or "danger," HSPs are not only increased in expression level (for the purpose of cytoprotection and antigen presentation) but could also undergo dynamic redistribution to gain access to the extracellular environment. Although the cell biological basis of extracellular distribution of HSPs is unclear, both the induction of HSPs and their cell surface expression or secretion might conceivably contribute to sending an "ON" signal to activate the immune system (Fig. 1) and hence to break down peripheral tolerance.

Concluding Remarks

Many surprising immunological features have been associated with HSPs since the original observation that HSPs can immunize against cancer in the absence of exogenous adjuvant. Collectively, it has raised several fundamental questions about HSPs: What is the source of extracellular HSPs *in vivo*? What is the physiological significance of HSPs in the immune response? What is the role of HSPs in immunological tolerance and memory? and What is the function of heat shock response in tumor surveillance? These questions have to be addressed in the context of stress responses and microenvironment of cancers. Answers to these questions have obvious implications both in terms of understanding the immune system but also in clinical translation of HSP technology for cancer immunotherapy.

In the meantime, there is a desperate need for well-designed clinical studies. Thus far, it is clear that customized HSP vaccination approach is feasible and relatively nontoxic. However, we have a long way to go before we know precisely how to use these remarkable molecules for battles against cancers.

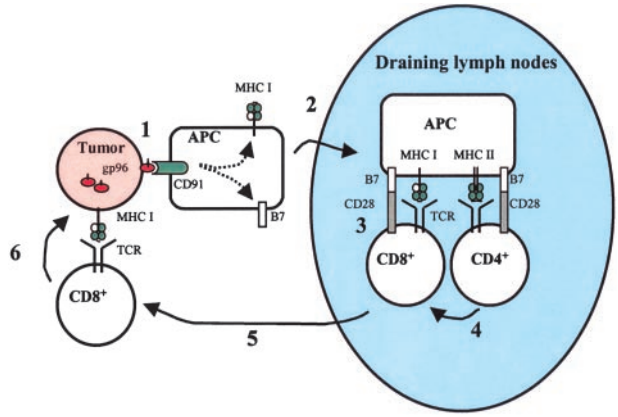

Fig. 1. HSPs such as gp96 prime antitumor immunity. Release of HSPs, such as gp96 because of stress or necrotic cell death, can serve as a pro-inflammatory signal. The interaction of gp96 with its specific receptors, such as CD91 on APCs (step *1*), leads to cross-presentation of antigens to MHC class I and up-regulation of costimulatory molecules such as B7. Activated DCs then migrate to the draining lymph nodes (step *2*), where they prime antigen-specific naïve T cells (step *3*). The activation of $CD4^+$ T cells can provide help for this process (step *4*). Those T cells then exit from lymph nodes (step *5*) into peripheral organs and tumor sites to elicit effector functions, *i.e.,* lysis and clearance of tumors in an antigen-specific manner (step *6*).

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