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COMMENTARY

Stress Protein/Peptide Complexes Derived from Autologous Tumor Tissue as Tumor Vaccines

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ABSTRACT. Vaccination of inbred mice with tumor-derived stress proteins hsp70, hsp90, and gp96/grp94 elicits a protective immunity to the tumor from which the vaccine was purified. There is now comprehensive experimental evidence that the antigenicity of tumor-derived hsp70, hsp90, and gp96 preparations results from diverse arrays of endogenous peptide antigens complexed with these stress proteins. Vaccination with tumor-derived stress protein/peptide complexes leads to their uptake and processing by professional antigen-presenting cells and to presentation of associated tumor peptide antigens to cytotoxic T cells. This induces a tumor-specific cytotoxic T cell response. The attractiveness of the concept of using tumor-derived stress proteins as vaccines is derived from two observations: (i) tumor stress protein vaccines mirror the individual antigenicity of a tumor, which results from random mutations due to genetic instability; and (ii) stress proteins represent powerful adjuvants for the peptide antigens complexed to them. BIOCHEM PHARMACOL **58**;9:1381–1387, 1999.

KEY WORDS. tumor immunology; heat shock proteins; T lymphocytes; tumor antigens; tumor vaccines; gp96

The induction of immunological control over malignant tumors by vaccination is the major research aim in tumor immunology. An important part of this research focuses on the activation of a specific T cell response against tumors. It is possible to induce a transplantation immunity in inbred mice that is strictly specific for the tumor that was used for vaccination [1–5]. By adoptive transfer experiments and *in vivo* depletion experiments it was shown that this tumor-specific transplantation immunity is mediated by T cells [6]. The growing knowledge of the molecular biology of cancer cells shows that cancer cells express a multitude of altered proteins due to mutations in the course of carcinogenesis and to the genetic instability of cancer cells. These alterations should be recognized by T cells of the tumor-bearing host as altered peptide epitopes, because it can be assumed that the host has not developed thymic tolerance toward these alterations. However, tumor development and growth are not under immunological control because tumor cells cannot efficiently activate T cells. In contrast to professional APC†, tumor cells do not express costimulatory molecules or a supportive cytokine milieu, which is necessary for efficient T cell activation [7]. On the contrary, tumor cells often secrete immunosuppressive cytokines such as transforming growth factor β, which promotes immune escape of the tumor [8], express apoptosis-inducing signals for T cells such as the CD95 ligand [9], and interfere with signal transduction in T cells [10]. Modern vaccination strategies against tumors try to circumvent these immunosuppressive mechanisms of tumor cells. One of these strategies is vaccination with subcellular material from tumor cells, which mirrors the individual antigenicity of the respective tumor. This approach employs professional APC for the induction of immunity. An autologous tumor vaccine should be manufactured from tumor cells by a standardized method and ideally should consist of defined cellular proteins. Experimental evidence shows that cellular stress proteins such as hsp70, hsp90, and gp96/grp94 and the endoplasmic chaperone calreticulin are complexed in cells with a diverse array of peptides including immunogenic peptide antigens [11–14] (Fig. 1). If these stress protein/peptide complexes are derived from tumor cells, they should represent at least a part of the individually distinct antigenic repertoire of the tumor. In consequence, they could be used as autologous tumor vaccines. In the following commentary, the experimental evidence for this concept of tumor-derived stress proteins as autologous tumor vaccines will be presented.

STRESS PROTEINS AS CHAPERONES FOR ENDOGENOUS PEPTIDES

Stress proteins are among the most abundant proteins in cells under physiological conditions because of their essential role as chaperones involved in protein transport and folding. Most stress proteins are expressed constitutively. Their expression can be enhanced under stress conditions

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† *Abbreviations:* APC, antigen presenting cells; CTL, cytotoxic T lymphocytes; hsp, heat shock protein; LCMV, lymphocytic choriomeningitis virus; MHC I, major histocompatibility complex class I; SV40, simian virus 40; TAP, transporter associated with antigen presentation; and VSV, vesiculostomatitis virus.

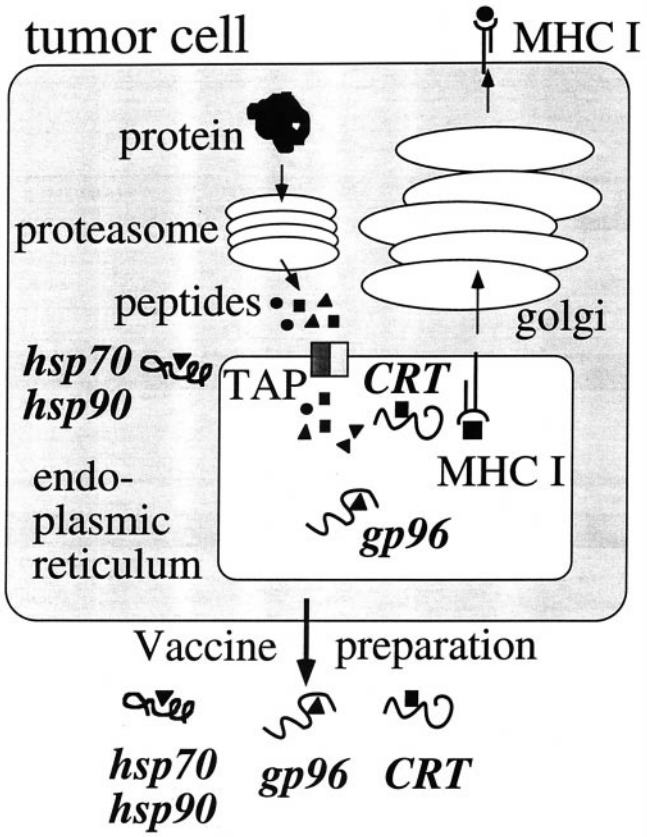

FIG. 1. Concept of tumor-derived stress proteins and chaperones hsp70, hsp90, gp96, and calreticulin (CRT) as autologous tumor vaccines. There is now comprehensive experimental evidence that the antigenicity of tumor-derived hsp70, hsp90, gp96, and calreticulin is derived from diverse arrays of endogenous peptide antigens complexed with these stress proteins. These peptide antigens are produced by proteasomal degradation of cytoplasmic proteins and can be complexed in the cytoplasm to hsp70 or hsp90. When peptide antigens are transported by TAP molecules into the endoplasmic reticulum, they either can directly associate with MHC class I molecules or can be bound by endoplasmic stress proteins or chaperones such as gp96 and calreticulin.

such as heat shock or glucose deprivation. Other stress proteins are inducible by stress conditions. There are several lines of evidence for the peptide binding functions of stress proteins hsp70, hsp90, and gp96, which are described below.

(i) The peptide binding function of stress proteins can be explained well by their functions under physiological and stress conditions. Under physiological conditions, stress proteins prevent immature folding of nascent proteins, assist intracellular protein transport through membranes, guide the assembly of protein complexes, and regulate function and degradation of cellular proteins. Under stress conditions, stress proteins prevent aggregation of partially denatured and misfolded proteins [15–18]. Due to these functions, stress proteins must have the ability to bind promiscuously to exposed peptide sequences of partially unfolded, misfolded, or nascent proteins.

(ii) The structures of stress proteins reveal possible peptide binding sites. Recently, the existence of a peptide binding pocket has been demonstrated in the hsp70 crystal structure [19]. Evidence for two substrate binding sites has been obtained for hsp90 [20].

(iii) Isolated stress proteins were shown to bind peptides *in vitro*. Hsp70 binds and releases peptides by cycles of ATP hydrolysis and ATP binding [21, 22]. Gp96 binds peptides independently of ATP hydrolysis in a temperature-dependent fashion, most likely by conformational change [23, 24]. Hsp70 and gp96 could be complexed with immunogenic peptide antigens *in vitro*. The reconstituted stress protein/peptide complexes elicited a peptide-specific CTL response in mice [23, 25].

(iv) Stress proteins were shown to bind peptides under physiological conditions. It was demonstrated recently by peptide translocation assays that gp96 is one of several peptide binding chaperones of the endoplasmic reticulum (ER) besides protein disulfide isomerase and calreticulin [26–28]. The peptide translocation in these studies was dependent on the function of TAP. Another study showed, by vaccination experiments with gp96 prepared from TAP-deficient or TAP-competent cells, that gp96 may associate with peptides whose presence in the ER is either TAP-dependent or TAP-independent [29]. Three studies obtained direct evidence for the association of naturally processed CTL-recognized peptide epitopes with hsp70 and gp96 in cells. Nieland *et al.* [30] reported the isolation of a naturally processed VSV-peptide epitope from gp96 preparations of VSV-infected cells, and Breloer *et al.* [31] showed the isolation of naturally processed ovalbumin epitopes from gp96 and hsp70 derived from ovalbumin-transfected cells. Ishii *et al.* [32] reported the isolation of an individual tumor peptide antigen from preparations of stress proteins hsp70, hsp90, and gp96 derived from a murine fibrosarcoma.

TUMOR STRESS PROTEINS AS TUMOR-SPECIFIC TRANSPLANTATION ANTIGENS

The important basis for the concept of tumor stress proteins as autologous tumor vaccines was the identification of stress proteins hsp70, hsp90, and gp96 as tumor-specific transplantation antigens of chemically induced mouse tumors [12, 33–35]. It was observed that the vaccination of inbred mice with tumor stress proteins elicited a strictly tumor-specific transplantation immunity against the syngenic tumor from which the stress proteins were derived. This individually distinct tumor immunity induced by tumor stress protein vaccination can be explained by an individually distinct array of immunogenic peptide antigens complexed with stress proteins in each tumor, since polymorphisms of stress proteins hsp70, hsp90, and gp96 are not known [36].

It was demonstrated that the priming phase of the immunization with tumor-derived gp96 was dependent on $CD8^+$ T cells and macrophages [37]. Immunization of mice with gp96 derived from UV-induced syngenic tumors elic-

ited tumor-specific CTL in parallel with tumor-specific transplantation immunity [38]. This suggests a model in which the vaccination with tumor-derived stress protein/peptide complexes leads to cross-priming of tumor-specific CTL by phagocytic APC. These APC must be able to take up and process the stress protein/peptide complexes and channel the peptide antigens into the MHC class I restricted presentation pathway.

The tumor immunity that is induced by tumor stress protein vaccination was demonstrated initially by preventive immunization against tumors. This seems not to be applicable in humans in view of the individuality and multiplicity of tumor antigens. However, it was shown recently that vaccination with tumor-derived gp96 and hsp70 also had therapeutic effects in early tumor growth and metastasis [39, 40]. So far, the applicability of tumor stress proteins as tumor vaccines has not shown restrictions concerning the type of experimental tumor, the histological origin, or the species. Tumor immunity induced by tumor-derived stress proteins was demonstrated against chemically induced tumors, UV-induced tumors, and spontaneous tumors of different histologic origins such as fibrosarcomas, lung carcinomas, melanomas, colonic cancers, and prostate cancers in mice and rats [33, 34, 38–41].

ACTIVATION OF ANTIGEN-SPECIFIC CTL BY ENDOGENOUS GP96/PEPTIDE COMPLEXES

In different antigenic systems the immunization of inbred mice with gp96 preparations elicited CTL responses against antigens of the cells from which gp96 was derived. This was shown for viral antigens from SV40 [42] and VSV [43], for influenza virus nucleoprotein [44], and for CTL-recognized model antigens such as β-galactosidase and minor histocompatibility antigens [45]. We investigated whether gp96 derived from human melanoma cells is associated with different CTL-recognized melanoma peptide antigens. It could be demonstrated that autologous human CTL clones specific for different melanoma peptide antigens were stimulated preferentially by gp96 derived from autologous melanoma cells compared with gp96 derived from autologous B cells. The CTL stimulation was dependent on monocytes or dendritic cells as APC. These results confirm for the first time in a human tumor model the association of gp96 with CTL-recognized tumor peptide antigens.*

There is no evidence that the spectrum of peptides associated with gp96 is dependent on the MHC haplotype of the cell. Therefore, gp96 as well as stress proteins hsp70 and hsp90 should be associated with peptides or precursors of peptides that differ in their binding motifs for MHC molecules and possibly can be presented by allogenic MHC molecules. This was confirmed by the observation that $CD8^+$ T cells of H-2b mice could be primed against minor H antigens by gp96 purified from H-2d positive cells [45], and VSV-specific CTL were induced in H-2b mice by gp96 preparations from H-2d VSV-infected cells [43]. This cross-priming potential by gp96 is important in view of shared tumor antigens, such as many CTL-recognized melanoma antigens. Individuals with different MHC haplotypes could possibly be cross-primed against shared tumor antigens by gp96 preparations from allogenic cell lines expressing these shared antigens.

ADJUVANT FUNCTION OF STRESS PROTEINS

Recently, it was shown that vaccination with *in vitro* reconstituted complexes of peptide antigens with gp96 or with hsp70 induces peptide-specific CTL responses and protective immunity *in vivo*. The induction of this immunity is dependent on binding of the peptide to the stress protein and was not induced by vaccination with peptide alone or mixtures of stress protein and peptide. Blachere *et al.* showed for different peptide antigens (viral and nonviral CTL epitopes) that vaccination with gp96/peptide complexes and hsp70/peptide complexes induced a peptide-specific CTL response, whereas vaccination with peptides alone or non-complexed mixtures of stress proteins and peptides did not. Additionally, the immunization with gp96/VSV-peptide complexes induced a protective immunity against a challenge with VSV-transfected tumor cells [23]. Ciupitu *et al.* showed that vaccination with hsp70/LCMV peptide complexes elicited LCMV-specific CTL and protective immunity against LCMV [25]. Vaccination with fusion proteins consisting of stress proteins and protein antigens seems to be an additional way to elicit antigen-specific CTL responses [46]. The mechanism of this adjuvant effect mediated by stress proteins is still unclear. Specific receptor-mediated uptake of stress protein/antigen complexes and primary activation of antigen-presenting cells by hsp70 and gp96 may play a role [14, 36, 43]. It is known from earlier work that hsp70 and hsp65 can serve as powerful adjuvants for protein and carbohydrate antigens in eliciting helper and humoral responses [47–51]. However, the recent work shows that stress proteins are natural adjuvants which channel bound antigens into the MHC class I presentation pathway, a very important observation for the development of vaccines against diseases that can be cleared by a cytotoxic T cell response. The attractiveness of stress proteins as adjuvants in contrast to conventional adjuvants lies in their lack of toxicity and in their potency to amplify both helper and cytotoxic T cell responses.

STRESS PROTEIN EXPRESSION IN TUMORS

Stress proteins confer on tumor cells an increased resistance against stress conditions such as hypoxia, acidosis, and glucose deprivation [52]. In consequence, overexpression of stress proteins by tumors is more likely than weak or lost expression. This hypothesis is confirmed by studies showing overexpression of constitutively expressed or inducible

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stress proteins of the hsp70 and hsp90 family in tumor tissue [53–55]. We found clear overexpression of gp96 in colorectal cancer cells compared with tumor stroma in 34 of 51 colorectal primary tumors and similar expression in cancer cells and stroma in the remaining 14 colorectal cancer specimens tested by immunohistochemistry. No loss of gp96 expression was observed in liver (N = 20) and lymph node metastases (N = 20). The transcription of gp96 in colorectal cancer cell lines was enhanced by glucose deprivation, not by heat shock, confirming the role of gp96 as a glucose-regulated stress protein in human colorectal cancer (Heike *et al.*, unpublished data). These results point to an essential role of gp96 in colorectal cancer cells in protection against hostile conditions of the tumor microenvironment, such as glucose deprivation. The protective role of tumor stress proteins is in agreement with the observation that increased expression of hsp70 and gp96 stress proteins is accompanied by increased tumorigenicity in mouse tumor models [56–60]. Furthermore, increased hsp70 and hsp60 expression in ovarian and breast cancer, respectively, has been associated with an unfavorable prognosis [61, 62]. However, the increased tumorigenicity of tumors induced by increased stress protein expression and the immunogenicity of tumor stress proteins are not contradictory. A correlation between immunogenicity of tumors and hsp70 expression was reported in two mouse tumor models [63, 64].

IMPLICATIONS FOR CLINICAL STUDIES

The advantage of tumor stress protein vaccines over vaccines consisting of defined tumor antigens lies in the observations that (i) tumor stress protein preparations mirror the individual antigenicity of a tumor due to multiple and random mutations; (ii) stress proteins represent powerful adjuvants for the peptide antigens complexed to them; and (iii) tumor-derived stress proteins represent tumor rejection antigens *in vivo*. The latter has not been shown for most CTL-recognized shared tumor antigens, which currently are being investigated in vaccine trials [65, 66]. The multivalent nature of tumor stress protein vaccines might prevent escape mechanisms of the tumor by antigen loss, as described after vaccination with single defined peptide antigens [67]. The preclinical data on the efficacy of tumor stress protein vaccines led to ongoing clinical phase I studies of tumor-derived gp96 preparations as autologous tumor vaccines in renal cancer, melanoma (M.D. Anderson Cancer Center, Houston, TX), pancreatic cancer (Memorial Sloan–Kettering Cancer Center, New York, NY), and gastric cancer (University of Mainz, Germany).

These trials will answer the question of whether this tumor vaccine strategy is feasible for different tumor entities. The stable and enhanced expression of gp96 in human tumors, as described for colorectal cancer, represents an important precondition for the feasibility of autologous gp96 tumor vaccines in clinical trials. The yield of gp96 from tumor tissue is also very important for this vaccine approach. From melanoma cell cultures approximately 30 μg of gp96/g of cell pellet can be purified by a standard method described in Fig. 2 (Heike *et al.*, unpublished observations). Gp96 preparations from tumor tissue give yields of between 15 and 150 μg/g of tissue [68]. In murine tumor models, the induction of immunity by gp96 vaccination requires 10–20 μg of gp96 when injected subcutaneously two times one week apart, and only 1 μg of gp96 two times one week apart in the case of intradermal injection [68].

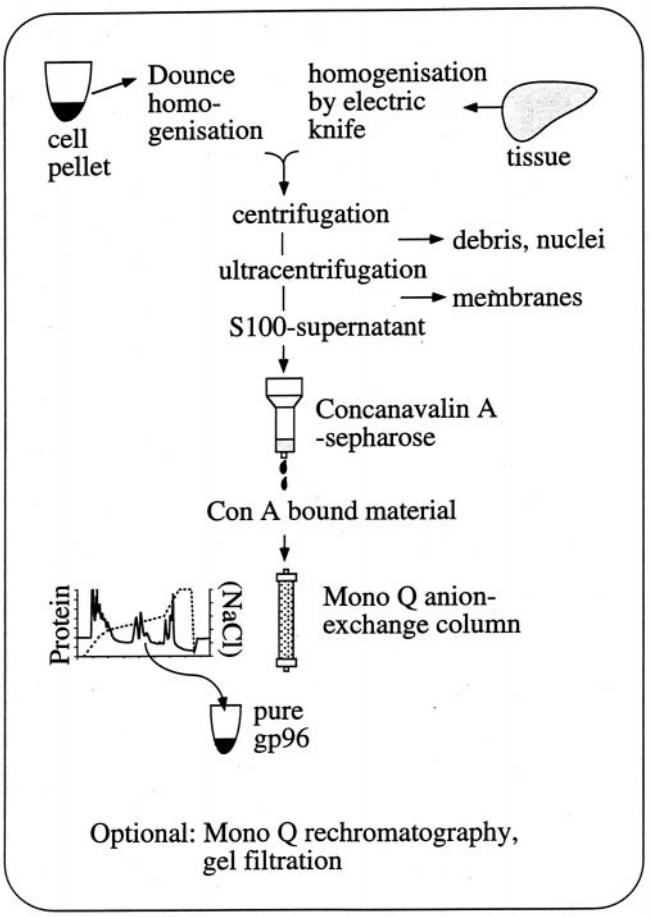

FIG. 2. Diagram of gp96 purification from cells or tissues by sequential column chromatography.

Another question of the trials will address the safety of stress protein vaccines. Since the endogenous tumor stress protein/peptide complexes certainly will contain peptides with normal sequences, the danger of causing an autoimmune reaction against these "self" epitopes is imminent. In mouse experiments no autoimmune reactions were observed [68]. However, this might be due to natural resistance of the investigated mouse strains against autoimmune diseases. In susceptible individuals thymic tolerance against "self" epitopes might not be complete, and peripheral tolerance might be broken by the strong adjuvant effect of stress proteins. Consequently, the clinical trials will need to monitor patients for autoimmune reactions carefully. In a

pilot trial, in which patients with end-stage tumor disease and different tumor entities were vaccinated with gp96 preparations derived from autologous tumor tissue, no autoimmune reactions or severe side-effects of the vaccine were observed [69].

A third question investigated by the clinical trials is whether the vaccination with autologous tumor-derived gp96 induces a T cell response in tumor patients against autologous tumor cells, or, in malignant melanoma, against known CTL-recognized tumor antigens. One endpoint of the trials is to find a vaccine dose that induces a T cell response, because this dose can be used in subsequent clinical efficacy studies. This endpoint is especially important in the gastric cancer and pancreatic cancer trials. In these trials, clinical efficacy cannot be assessed easily, because the trials include patients who underwent surgery with curative intent, although these patients have a high risk of relapse. In murine tumor models, the dose–response relation of gp96 vaccination can show a bell-shaped curve [33, 70]. It is not possible to extrapolate the efficient gp96 vaccine dose for humans from the mouse experiments. Consequently, a broad dose range has to be tested in the first trials of gp96 vaccination. The study of gp96 vaccination against pancreatic and gastric cancer in an adjuvant setting has the potential advantage that vaccination against tumors may work better with minimal residual disease than with a high tumor burden. A risk of these trials might be that the anti-tumor T cell reactivity induced by the vaccine might not reflect clinical efficacy. The induction of a T cell response against tumors is not necessarily accompanied by tumor regression in murine tumor models [71].

There are still a number of open questions concerning the mechanisms at work in stress protein vaccination. It is unclear how stress protein/peptide complexes are incorporated and processed by APC, how associated peptide antigens enter the MHC class I restricted antigen presentation pathway, and which APC are effective. It is also unclear how representative are the tumor antigens that are complexed with the respective stress proteins. It can be assumed that hsp70, hsp90, and gp96 are complexed with different repertoires of peptide antigens due to their specific subcellular locations and different binding properties. Peptide motifs of endogenous peptides complexed with the different stress proteins have not been published thus far. The answers to these questions will lead to further vaccine strategies. The problem of the bell-shaped dose–response curve for gp96 vaccination could be overcome by vaccination with gp96-pulsed professional APC-like dendritic cells, once they have been shown to process gp96/peptide complexes and present the associated peptides. Since cytoplasmic hsp70 and hsp90 are even more abundant than gp96 and possibly differ in their repertoire of associated immunogenic peptides, one can envision vaccinating patients with a panel of different stress proteins purified from autologous tumor tissue.

NOTE ADDED AT PROOF

Concerning the adjuvant function of stress proteins, a recent report described receptor-mediated endocytosis of stress proteins gp96 and hsp70 by murine APC (Arnold-Schild D, Hanau D, Spehner D, Schmid C, Rammensee H-G, de la Salle H, Schild H, Receptor-mediated endocytosis of heat shock proteins by professional antigen-presenting cells. *J Immunol* **162:** 3757–3760, 1999). Two additional phase I studies with tumor derived gp96 preparations as autologous tumor vaccines have started in melanoma and colorectal cancer (Istituto dei Tumori, Milan, Italy).

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