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A role for the Heat Shock Protein-CD91 axis in initiation of immune responses to tumors

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Abstract

For over a hundred years, it has been established that tumor-specific immune responses can frequently be detected in the tumor bearing host. Whether or not these immune responses are capable of controlling the growth of the tumor is influenced by many factors. However, the mechanism by which the immune responses are initiated in the first place has remained a dilemma. In this chapter, we present evidence that heat shock protein-peptide complexes released by tumor cells are the entity responsible for initiating the immune responses. Interaction of the extracellular HSP with its receptor CD91 is necessary for priming the immune response. We propose that the disruption of the HSP-CD91 interaction may be an active mechanism by which tumors prevent the generation of immune responses against it.

Keywords

Heat shock protein; CD91; tumor immunity; concomitant immunity

Introduction

All tumors have some degree of immunogenicity, thereby capable of priming immune responses in the bearing host. Why then do tumors grow? From the onset, immunogenicity has to be distinguished from immunosensitivity, the ability of the immune response of some effector type to reject the tumor. A variety of mechanisms have been described by which tumors become insensitive to immune responses. Thus, while most tumors elicit immune responses, a smaller percentage will actually be rejected. In this chapter, we describe a mechanism by which a growing tumor elicits specific immune responses in the bearing host.

The ability of tumors to elicit immune responses was first described by Ehrlich, P., in 1906 (1). The term concomitant immunity was coined a few years later by Bashford, E., (2) to describe the phenomenon whereby mice carrying a progressively growing tumor implant would reject a second implant of the same tumor. With the advent of inbred mice in the 1950s, the contribution of concomitant immunity to tumor immunity could finally be established without interference of haplotype rejection. Robert North elegantly performed these experiments with several different tumor models in the 1970s (3). Tumor immunity and, when it occurs, tumor rejection, is primarily a T cell mediated response; T cells will transfer immunity (4) and depletion of T cells from mice abrogates tumor immunity (5). The starkly apparent question that has gone unanswered for decades is; what exactly in the tumor cells is responsible for priming T cell immune responses? Firstly, there must be some contribution of components of the antigen which is responsible for the specificity of the

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response and, secondly, an initiator of innate signals commonly called co-stimulation. These two signals are necessary for priming T cell immune responses (6). In this chapter, we will present evidence and a discussion that these two signals do not come separately but are contributed by the tumor cell as a single entity; the heat shock protein-peptide complex.

Heat shock proteins elicit T cell responses

A search for tumor rejection antigens in the 1980s was approached from several different directions. One of these approaches was the biochemical fractionation of tumor cells which led to the identification of several heat shock proteins (HSPs) as tumor rejection antigens (7). Generally HSPs are classified into different families based on their sequence homology. They are abundant intracellular proteins and several of them act as chaperones of proteins, peptides, lipids and nucleic acids within various compartments of the cell. Gp96, an endoplasmic reticulum protein, was the first HSP shown to have immunological properties followed by its cytosolic paralogue hsp90 and hsp70 (8). Calreticulin (9), hsp110 (10) and grp170 (10) complete the list of HSPs to date that have immunological properties. As tumor rejection antigens, these HSPs, purified to apparent homogeneity from tumor cells were able to protect immunized mice from a subsequent challenge with live tumor cells. Interestingly, HSPs purified from irrelevant tumors or from normal tissue failed to confer protection (7-9). The observed specificity of immune responses elicited with tumor-derived HSPs was unexpected given that the tumor-derived HSPs harbored no (tumor-specific) mutations when aligned with normal tissue-derived HSPs, neither on a nucleic acid nor amino acid sequence level (11).

Clues to the origination of specificity arose from the unique ATP/ADP-dependent association of ligands to hsp70. Treatment of hsp70 with ATP removed associated peptides while simultaneously abrogating the immune protective effects (12,13). Those experiments strongly suggested that the associated peptides were responsible for the specificity of the immune responses elicited and not the HSP per se. Experimentally, this was demonstrated in various other ways; i) peptides can be bound to HSPs *in vitro* by mild temperature elevation to form complexes which can elicit immune responses with specificity for the bound peptide (14), (ii) peptides can be stripped off the HSPs with several reagents to render the HSP non-immunogenic (13), and (iii) recombinant HSP-peptide fusion molecules are immunogenic, again with specificity directed to the bound peptide (15,16). Interestingly, the peptide has to be associated with the HSP either covalently or non-covalently for immunogenicity; simply mixing the HSP with peptide does not confer immune properties (14). Thus immune responses elicited by tumor-derived HSPs will be directed to the tumor specific antigens and so on for HSPs purified from viral (17) or bacterial infected cells (18). Alternatively, one can pick an antigenic peptide of choice, complex it to HSPs and elicit immune responses with a defined specificity (14,19). HSPs generally do not discriminate in the peptides they chaperone and do chaperone self peptides too. The question of autoimmunity has therefore arisen. However, in all the experimental systems in frogs, mice and humans, no autoimmunity has been detected even when critically examined.

The immune responses elicited with HSP-peptide complexes has far reaching consequences ranging from basic immunology where HSPs are now being called Danger Associated Molecular Patterns (DAMPs) to immunotherapy of cancer and infectious diseases where HSPs are currently in clinical trials (20-23). The work described above shows that the HSP-peptide complex as an entity is capable of eliciting immune responses upon immunization. Later on, we will present evidence that the same HSP-peptide complexes when released from tumor cells growing *in vivo* is capable of the same.

The HSP receptor, CD91, is critical for HSP mediated immunity

By depleting various cellular subsets in mice, tumor-specific immune responses elicited by HSPs were shown to be dependent on T cells and antigen presenting cells (APCs) (24). There was no significant contribution by B cells in the tumor setting. Priming of T cells invokes a key player, the antigen presenting cell (APC). Early studies examined the effects of HSPs on APCs and the proposal for the existence of an HSP receptor grew from the following observations (25-26); (i) HSPs interacted with APCs in a specific, titratable and competent manner, (ii) effective immunization of mice against a tumor challenge required minute quantities of HSPs indicating a highly efficient mechanism, and (iii) incubation of APCs with HSPs led to the internalization of the HSP. Cross-linking and chromatographic experiments with APCs *in vitro* isolated CD91 as a receptor for the HSPs, gp96, hsp70, hsp90 and calreticulin (27). This observation has been corroborated by genetic, immunological and biochemical studies in numerous laboratories conducted both *in vitro* and *in vivo* (28-36). The emphasis on this large body of work is purposeful due to a number of other proposed HSP receptors with feeble and inadequate support, often confused with effects of contaminating LPS (reviewed in 37). Cross-presentation assays established *in vitro* have demonstrated that peptides chaperoned by HSPs are internalized in a CD91-dependent manner and processed and presented on MHC I and MHC II molecules (27-31,38-42). Thus APCs treated with HSP-peptide complexes are capable of stimulating their corresponding CD8⁺ and CD4⁺ T cells respectively. The processing mechanisms for both pathways have largely been worked out (43). The role of CD91 *in vivo* has been demonstrated. Co-immunization of mice with HSPs and anti-CD91 antibodies abrogates immunity and thus these mice no longer reject a tumor challenge. In addition to cross-presentation, CD91 has recently been shown to be involved in signaling within the APC culminating in activation of co-stimulation by way of cytokines and co-stimulatory molecule expression (44). The specific (cytokine) signals contributed by the APCs in response to HSPs skew T cells responses to the Th1 (24), Th2 (17), or Treg (45) phenotype.

The HSP-peptide complex offers the perfect regimen for initiation of specific immune responses against tumors once released from their intracellular compartments. HSPs are found in the extracellular environment when plasma membranes of tumor cells are compromised (46). There have also been several active secretory mechanisms proposed, although this remains to be entrenched experimentally (48). In the extracellular environment the HSPs are available to engage APCs in the local environment or drain in the lymphatics and engage APCs in the lymph nodes. These various routes are being investigated to find the APC subtype(s) responsible for cross-priming with HSP-peptide complexes (Figure 1). We have used fluorescent labeled gp96 to track the HSPs *in vivo* in order to identify the cells that interact with it for cross-priming. An intradermal injection of a few micrograms of gp96-A₄₈₈ shows a dramatic labeling of CD11b⁺ and to a lesser degree, CD11c⁺ cells in the draining lymph nodes an hour later. The labeled cells represent less than 1.0% of the total draining lymph node cells. We are in the process of identifying these cells as far as APC subsets is concerned and also isolating these cells to characterize their functional significance for cross-priming. From preliminary studies, a summary of the subtyping of the gp96⁺ cells derived from draining lymph nodes is shown in Figure 1. Most of the facets of the responses elicited by HSPs have been studied by immunizing mice with a few micrograms of HSPs. While this data is certainly very informative it suggests but not prove that HSPs released from tumor cells are responsible for initiation of anti-tumor immune responses. Those experiments are described in the next section.

Abrogating the HSP-CD91 interaction inhibits immune responses

We have previously tackled this issue in one experimental approach by depleting tumor cell lysates of various components to determine immunogenic molecules. Depletion of the dominant HSPs abrogated the ability of cell lysates to prime immune responses even though the antigenic proteins were still present (48). Conversely, depletion of antigenic proteins had no effect on the ability of tumor cell lysates to prime. To address the specific role that CD91 plays in the initiation of immune responses we decided to use the strategy of tumor cells transfected with an inhibitory protein that would block the association of HSPs with CD91. The Receptor Associated Protein (RAP) binds with high affinity and prevents the association of all known ligands to CD91 (for review see 49). Specifically RAP has been shown to effectively inhibit the binding of HSPs to CD91 thereby inhibiting cross-presentation of the chaperoned peptide (29). The hypothesis was that by expressing RAP constitutively, tumor cells would release RAP into the same microenvironment as the HSPs, inhibit HSP-mediated cross-priming and abrogate immune responses to that tumor. By doing so these tumors should be less immunogenic and grow with faster kinetics. These experiments have been performed and the preliminary data shows that indeed, expression of RAP by tumor cells decreases their immunogenicity and hence have a faster rate of growth compared to the control transfected tumor cells (Figure 2). We have also determined that transfection of RAP in tumor cells itself does not have any effect on rate of proliferation of the cells. These studies heavily support the idea that the HSP-CD91 interaction is important for mounting anti-tumor immune responses.

Other cellular components that activate APCs

While we have focused entirely on HSPs and the receptor CD91, a word must be mentioned about other tumor derived molecules that have been shown to have some immune component. Molecules such as HMGB1 (50), double stranded DNA (51) and uric acid crystals (52) have been shown to stimulate DCs to produce cytokines. For some, such as HMGB1, cell surface receptors (Rae1) have been described. To this end, the requirement for co-stimulation is satisfied but these “DAMPs” do not provide tumor specificity in terms of the antigen (signal 1). The antigen by itself, either as a whole protein or a peptide derivative has been strongly argued to be insufficient in quantity to contribute signal 1 (48,53). In fact the typical amount of antigen produced by a tumor cell is 50,000 times less than is required for priming an immune response. Thus there is a requirement for a high efficiency process for cross-presentation such as that provided by the HSP. The HSP increases cross presentation of peptides by several orders of magnitude over the antigen by itself. This has been painstakingly demonstrated experimentally. The non-HSP DAMPs are therefore insufficient in priming tumor-specific immune responses but may however contribute to it by providing signal 2.

Final remarks

The future in studying the HSP-CD91 axis lies in the creation of mice deficient in expression of the various players. HSP deficient mice are non-viable due to the indispensable role that these HSPs play. Conventional CD91 knock-out mice are also embryonically lethal but it is possible to generate conditional knock-out mice using the cre-lox system. Thus CD91 expression can be selectively eliminated in APCs. The important question is which APCs to target? Our studies to date have identified both a CD11b⁺ and CD11c⁺ APC population in the draining lymph nodes and these would be the logical choice of cells to targets. Those studies have been corroborated by cross-presentation *in vivo* and *in vitro*. Although cross-priming has been a bona fide and exclusive property of dendritic cells (DCs) characterized by their expression of CD11c and/or CD11b (54), there are however a

few notable exceptions in certain macrophages subsets for example (55). Mice deficient in CD91 expression in the relevant APCs can then be tested for their ability to mount immune responses against a tumor challenge, either in a concomitant tumor rejection assay or by active immunization with irradiated tumor cells.

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Biography

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b.

Phenotype of gp96 ⁺ cells after intradermal administration.	
Surface Markers	Percent of gp96 ⁺ cells
CD11c-CD11b+	27.6%
CD11c+CD11b+	59.0%
CD11c+CD11b-	2.3%
CD11c-CD11b-	11.1%

Figure 1.

The fate of extracellular gp96. (a) Extracellular gp96 in the dermis can be introduced by tumor cells or through active immunization and it can have several fates. Gp96 can be taken up by non-migratory APCs (1) and remain in the dermis. Gp96 can drain passively to the lymph nodes (2) and be taken up by lymph node resident APCs (4). Gp96 can be taken up by dermal APCs which would then migrate to the lymph node (3). In the lymph node, migratory APCs may directly interact with T cells or transfer the HSP to an adjacent and lymph node resident APC (5). (b) Phenotype of gp96⁺ cells from draining lymph nodes after an intradermal administration of 10 μ g of gp96-A₄₈₈.

Figure 2. RAP inhibits tumor immunogenicity. Cohorts of BALB/c mice were immunized with CMS5 cells transfected with RAP or control plasmid. The average tumor diameter is shown (mm) on day 15 after inoculation of 1×10^6 cells. * $p < 0.005$