Heat shock proteins refine the danger theory

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Evidence is mounting to support the theory that 'danger' associated with tissue damage and stress is a level of control of antigen-specific immune responses. Heat shock proteins are emerging as key danger signals, but their influence on immune responses is not a simple one, particularly in affecting antigen processing and presentation. Such influences may extend to numerous immunological situations. Potential exists for heat shock proteins to be utilized in immune modulation, especially in cancer immunotherapy.

INTRODUCTION

Deficiencies of the self–non-self paradigm in explaining immune phenomena, such as the existence and breaking of immune tolerance, have lead the likes of Janeway[1] and Matzinger[2] to suggest additional levels of control of antigen-specific immune responses. In simple terms, the immune system is thought to be turned on by 'danger' associated with certain molecules of infectious organisms, or cell products released during tissue damage or stress. Antigen-presenting cells (APC) possess pattern-recognition receptors that can recognize such molecules. This recognition is thought to cause upregulation of costimulatory molecules on local APC, which is the second signal [in addition to antigenic peptide with major histocompatibility complex (MHC)] that is required to initiate an antigen-specific immune response. Candidate danger signals from infectious organisms include lipopolysaccharide, mannans, glycans and CpG DNA motifs, whilst those from mammalian cells include heat shock proteins (hsp; inducible and constitutive), mitochondria, mannose, RNA and DNA. With respect to hsp, recent evidence now suggests that their role in signalling danger may be wide ranging and, not surprisingly for immunology, more complicated than first thought.

HEAT SHOCK PROTEINS

Initial immunological interest in hsp surrounded the concept of hsp being self antigens that are highly conserved throughout evolution and that cross-react with microbial hsp, giving rise to autoimmunity.[3,4] This applied to diseases such as arthritis and diabetes. Microbial hsp were also thought to contribute adjuvant properties to vaccines, e.g. mycobacterial hsp70.[5] More recently, hsp as antigens have been implicated in allograft rejection.[6] The normal functions of hsp include binding and transporting of proteins in the endoplasmic reticulum and cytoplasm, whilst ensuring their correct folding.[7] Thus, a role in normal antigen processing and presentation has been suggested.[8] The antigen-chaperoning properties of hsp were highlighted in the tumour vaccination setting by the work of Srivastava (reviewed in 9). A number of hsp have now shown the ability to carry tumour antigens and generate protective immunity against live tumour challenge in murine models, including hsp70, hsp90 and gp96. In these experiments the hsp molecules were extracted and purified from tumours against which immunity was sought. Hsp from normal tissues or hsp from tumours, which were then stripped of their associated peptides by adenosine triphosphatase (ATPase) before vaccination, did not confer protection against tumour.[10,11] Vaccination was very efficient, requiring only nanogram quantities of soluble hsp–peptide complex. Indeed, large amounts of hsp complex could induce immune tolerance.[12] Hsp were also shown to transfer viral antigens,[13] minor histcompatibility antigens and model antigens,[14] thus generating immunity. In addition, hsp–peptide complexes capable of eliciting immunity *in vivo*, have been generated from hsp and peptide mixed together *in vitro*.[15] It was demonstrated that hsp complexes bind to professional APC via surface receptors, are taken up into the APC, and the peptides processed and presented with MHC class I.[10,11] Thus hsp allow exogenous antigen access to the endogenous antigen-processing pathway, i.e. they promote antigen cross-priming. These findings were recently confirmed visually using electron microscopy.[16]

DEATH OF A CELL

The danger theory suggests that the mechanism by which a cell dies will dictate whether an immune response is initiated.[2] Necrotic cell death and lysis often occurs during viral or bacterial infection, tissue damage and acute inflammation; situations where immune responses are beneficial and even critical to survival. Mechanisms of cell death, however, are often not clear cut and we have discussed these processes in more detail elsewhere.[17] The upregulation of hsp in response to cell stress was initially described as a means of cytoprotection, where unfolded cell proteins are bound and protected from stress-induced damage (reviewed in 18). This response to stress

'Dangerous' cell death

'Quiet' cell death

Tissue damage/stress
Viral infection
Inflammation

↑hsp

T cells feed
back to kill
tumour cells

Stress-associated cell death/lysis
Necrosis
Danger signals (including hsp)

hsp

hsp   hsp   hsp

Many DC recruited and
efficiently take up
tumour antigen

DC mature and migrate to lymph nodes

CTL

T helper

Cytokine
help

Normal cell turnover
Cytotoxic cell mechanisms

Apoptosis

Macrophage phagocytosis

No danger signal

No costimulation

T-cell anergy/
death

IL-10

● antigen
▬ costimulatory molecule (B7)
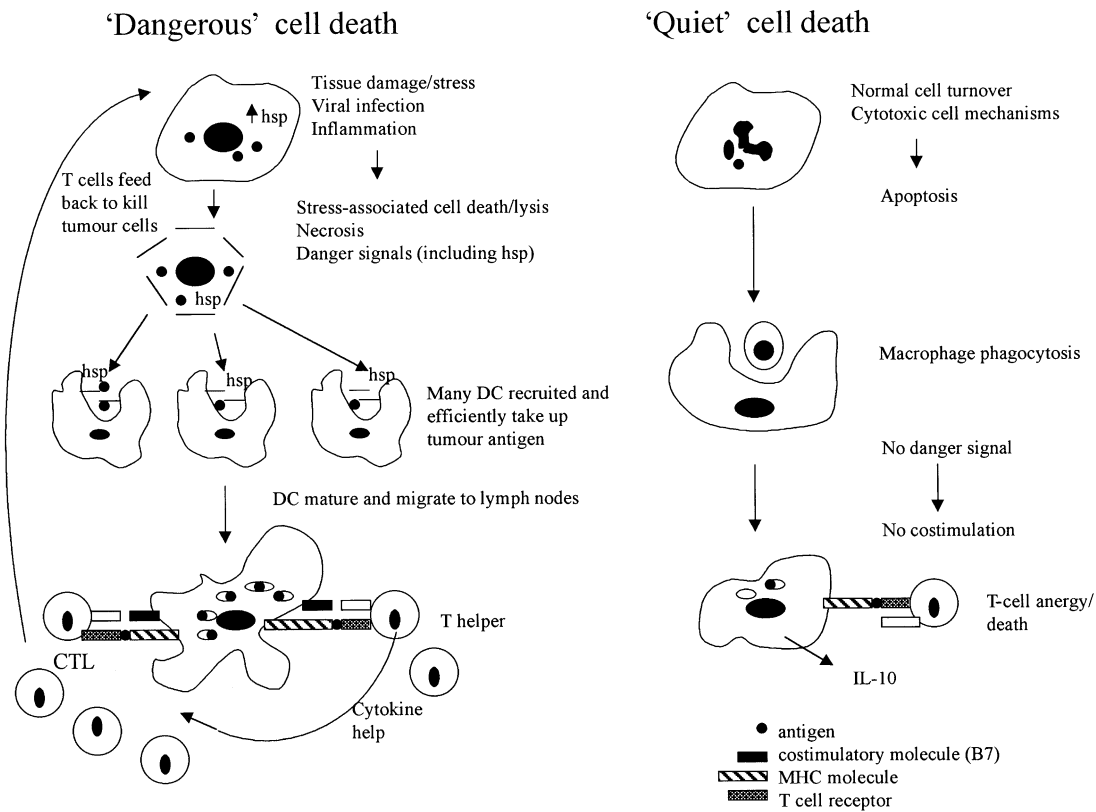▨ MHC molecule
▦ T cell receptor

Figure 1. Summary of immune reactions to 'dangerous' and 'quiet' cell death. Cells dying by apoptotic, 'quiet' cell death are scavenged by macrophages. Peptides are presented in the absence of costimulation and T cells die or are anergized. Cells dying in 'dangerous' situations, induced by tissue damage/stress (physical/chemical), viral infection or inflammation (leading to necrotic cell death), cause danger signal upregulation and/or release, and cell lysis. DC are recruited in numbers and efficiently take up antigens, especially antigens chaperoned by hsp. DC mature and migrate to lymph nodes where they present peptides to T cells with co-stimulation. T cells primed/activated in numbers can feed back and kill tumour cells.

is used by a wide range of organisms. It has been postulated that a response to released hsp (constitutive or induced) may be an evolutionary remnant from primitive ancestral immunity.[9] It makes sense that hsp could be a first line signal to the innate immune system against danger. In our recent studies we showed that when certain tumour cells were killed by a non-apoptotic (necrotic) mechanism (mediated by thymidine kinase and ganciclovir) they expressed raised levels of inducible hsp70, and other hsp.[19] Such killing *in vivo* gave rise to immune protection against subsequent tumour challenge. It is an accepted concept that normal cell turnover must not induce an (auto-) immune response, and apoptosis generally ensures this, as cell contents are not released ('quiet' death). Whole apoptotic cells are scavenged by macrophages, which present peptides in the absence of co-stimulatory molecules since no danger is perceived (Fig. 1). In addition, studies suggest that apoptotic cells can be actively inhibitory to immune responses, e.g. by eliciting interleukin-10 (IL-10) production by macrophages.[20] However, other work has shown that antigen from apoptotic cells can be efficiently presented under appropriate (often *in vitro*) conditions.[21] We found that certain tumour cells that died by thymidine kinase-induced apoptosis did not induce hsp and were not immunogenic *in vivo*. However, co-transfection with apoptosis-blocking bcl-2 drove these cells towards necrosis, accompanied by hsp expression and immunogenicity. We found that this hsp expression occurred *in vivo*

and *in vitro* and so was not primarily influenced by other cell types. Interestingly, tumours expressing cytokines [IL-2, granulocyte–macrophage colony-stimulating factor (GM-CSF)] expressed hsp *in vivo* but not *in vitro*,[19] suggesting hsp mediation by inflammatory cells. When tumour cells were transfected to overexpress hsp 70 they were equally as immunogenic as during necrotic cell death. Thus, hsp expression could replace the necrotic mechanism in generating immunity.

A number of studies describe the upregulation of hsp in response to adenoviral infection,[22] and during inflammation associated with allograft rejection[6,23] and rheumatoid arthritis.[24] The mechanisms behind this upregulation remain to be deciphered. It is tempting to suggest that viral-, stress- or inflammation-mediated hsp expression may be involved in self antigen transfer to APC and cross-priming during autoimmunity or graft rejection. Although these conditions tend to be multifactoral, such a process may have a precipitating or potentiating role.

REFINING THE DANGER THEORY

A number of reactions must coincide to generate an antigen-specific immune response in a 'dangerous' situation. Evidence is now growing that hsp may have numerous effects on shaping antigen-specific immune responses. Recently, it has been

demonstrated that hsp can directly induce macrophages[25] and cytotoxic T cells[26] to secrete inflammatory cytokines such as tumour necrosis factor-α (TNF-α). However, it appears that simply triggering dendritic cell (DC) maturation may in itself not be the ideal mechanism for inducing immunity. Indeed, it is logical that the DC needs to take up antigen before maturation and antigen presentation. In a follow up to our above studies[19] we found that radio-labelled lysates from tumour cells over-expressing hsp70 were taken up by immature DC *in vitro* to a far greater degree than lysates of unmodified cells.[27] This effect was lost when mature DC were used. Tagged hsp70 showed that the DC specifically took up the molecule. Incubation of DC with lysates from unmodified cells caused far greater MHC class I and II upregulation than hsp-cell lysate, suggesting reduced maturation when hsp was present. *In vivo*, hsp-expressing tumours were heavily infiltrated with DC. However, mixed cell tumours of hsp-expressing and unmodified tumour cells only elicited protection against the unmodified cell line that had been expressing hsp. Thus, hsp derived from stressed cells appear to combine the properties of being able to efficiently and specifically transfer antigen to APC,[1] and recruiting and maintaining APC in an antigen-acquiring condition (Fig. 1).[2] This may be preferable for presenting peptides that are less favourably presented or are in low quantity (causing epitope spreading). Peptides that are easily presented are more likely to have caused tolerance of T cells peripherally or during ontogeny, depending on the antigen. Inducing hsp expression may, in particular, be beneficial in overcoming immunosuppressive mechanisms in the tumour or tumour-vaccine microenvironment, in addition to breaking tolerance to tumour antigens. On the other hand, hsp expression in certain circumstances may induce unwanted autoimmune responses.

CONCLUDING REMARKS

Greater flexibility in the way that immunologists view their field, together with mounting evidence, has resulted in the general acceptance of the danger hypothesis in influencing or controlling antigen-specific immune responses. Heat shock proteins are implicated as possible danger signals, but their influence may be more subtle and more wide-ranging than first thought. The potential clearly exists for hsp to be manipulated in immunological settings. This is particularly relevant in cancer immunotherapy, since hsp can make tumours appear dangerous to the immune system,[28] and can transport tumour antigens for efficient T-cell priming.

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