Mechanisms of HSP72 release

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Currently two mechanisms are recognized by which heat shock proteins (HSP) are released from cells; a passive release mechanism, including necrotic cell death, severe blunt trauma, surgery and following infection with lytic viruses, and an active release mechanism which involves the non classical protein release pathway. HSPs are released both as free HSP and within exosomes. This review covers recent findings on the mechanism by which stress induces the release of HSP72 into the circulation and the biological significance of circulating HSP72 to host defense against disease.

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1. Introduction

Heat shock proteins (HSP) were originally described for their roles as chaperones induced by temperature shock as well as various other kinds of stress including environmental (UV radiation, heat shock, heavy metals and amino acids), pathological (bacterial, parasitic infections or fever, inflammation, malignancy or autoimmunity) or physiological stresses (growth factors, cell differentiation, hormonal stimulation or tissue development), that induced a marked increase in intracellular HSP (iHSP) synthesis known as the stress response (Lindquist and Craig 1988). This is achieved by activating the trimerization and nuclear translocation of cytoplasmic heat shock factor-1 (HSF-1) for binding with the heat shock elements (HSE) and consequent transcription of *HSP* genes. By binding unfolded, misfolded or mutated peptides or proteins and transporting them to the endoplasmic reticulum (ER), HSP prevent potential aggregation and/or death. Within the ER the peptides are released in an ATP-dependent fashion and refolded (Fink 1999). Recently an additional role has been ascribed to HSP as danger signals produced and released when cells are under stress and as activators of the immune system. This review will focus mainly on the HSP70 family, which constitutes the most conserved and best studied class of all heat shock proteins. The human HSP70 family consists of at least 12 members (for review *see* Tavaria *et al* 1996). The best known HSP70 family members are the constitutively expressed HSP70 (Hsc70 or HSP73; molecular weight of 73 kDa), the stress inducible HSP70 (HSP70 or HSP72; molecular weight of 72 kDa), the mitochondrial HSP70 (HSP75; molecular weight of 75 kDa), and the endoplasmic reticulum HSP70 (Grp78; molecular weight of 78 kDa). This review will briefly delve on recent advances in our understanding of how stress induces the release of HSP72 into the circulation. I will also discuss the steps required for initiation of an immune response and its biological significance in the context of host defense.

2. Chaperokine activity of extracellular HSP72

Chaperokine, is a term recently coined to better describe the unique function of extracellular HSP72 (eHSP72) as both

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Abbreviations used: APC, antigen presenting cells; eHSP72, extracellular HSP72; Hsc73, constitutive seventy-kilo Dalton heat shock protein; HSP72, inducible seventy-kilo Dalton heat shock protein; IFN-γ, interferon-gamma; iHSP72, intracellular HSP72; TLR, toll-like receptors

chaperone and cytokine (Asea 2003 2005). The consequence of binding and signaling is the stimulation of a potent and long lasting immune response. eHSP72 induces a plethora of immune responses and the list continues to grow. Briefly, as early as 2–4 h post exposure of APC to eHSP72, there is a significant release of cytokines including TNF-α, IL-1β, IL-6 and IL-12 (Asea *et al* 2000a,b), GM-CSF (Srivastava 2002); nitric oxide, a potent apotogenic mediator (Panjwani *et al* 2002); chemokines including MIP-1, MCP-1 and RANTES (Lehner *et al* 2000; Panjwani *et al* 2002). This part of the immune response does not require peptide, since both peptide-bearing and non peptide-bearing eHSP72 is capable of inducing pro-inflammatory cytokine production by APCs (Asea *et al* 2000a). However, peptide is required for specific $CD8^+$ CTL responses (Srivastava *et al* 1994; Srivastava 2000, 2005). eHSP72 induces the DC maturation by augmenting the surface expression of CD40, CD83, CD86 and MHC class II molecules on DC (Basu *et al* 2000; Singh-Jasuja *et al* 2000; Asea *et al* 2002a; Noessner *et al* 2002) and migration of DC (Binder *et al* 2000) and NK cells (Gastpar *et al* 2005).

2.1 *Dichotomy of HSP70 effects*

A dichotomy now exists between the effects of HSP based on its relative location, intracellular versus extracellular, and the target cell it binds to and activates. The upregulation of intracellular HSP70 (iHSP72) is generally cytoprotective and induces the cell's anti-apoptotic mechanisms (Jaattela *et al* 1998), represses gene expression (Tang *et al* 2001), modulates cell cycle progression (Hut *et al* 2005) and is anti-inflammatory (Housby *et al* 1999). On the other hand, the upregulation of extracellular HSP70 (eHSP72) is generally immuno-stimulatory and stimulates pro-inflammatory cytokine synthesis (Asea *et al* 2000c; Asea 2005), augments chemokine synthesis (Lehner *et al* 2000; Panjwani *et al* 2002), upregulates co-stimulatory molecules (Asea *et al* 2002b; Bausero *et al* 2005b) and enhances anti-tumour surveillance (Srivastava *et al* 1994; Srivastava 2000, 2005).

3. Passive release mechanism

Gallucci and colleagues initially demonstrated that dendritic cells (DC) are stimulated by endogenous signals received from stressed, viral-infected or necrosis-induced cells, but not by healthy cells or cells undergoing apoptosis (Gallucci *et al* 1999). In a series of elegantly performed experiments, Basu and co-workers later reported that heat shock proteins including gp96, calreticulin, HSP90 and HSP72 are released from cells by necrotic but not apoptotic cells (Basu *et al* 2000). These authors demonstrated that necrosis induced by freeze thaw, but not apoptosis induced by irradiation, resulted in the release of HSP into the culture supernatant (Basu *et al* 2000; Basu and Srivastava 2000; Srivastava and Amato 2001; Srivastava 2003). During apoptotic cell death, the contents of the cell are not released into the external milieu but are packaged neatly into apoptotic bodies, which are efficiently scavenged by neighbouring professional phagocytes. However, necrotic cell death results in the discharge of intracellular contents into the extracellular milieu thereby liberating heat shock proteins (for review *see* Srivastava 2003; Calderwood 2005). These results make the necrosis hypothesis an attractive explanation for the mechanism by which heat shock proteins are released into the circulation.

A condition in which necrotic cell death clearly contributes to the release of HSP72 from cells is following severe trauma. Pittet and colleagues (Pittet *et al* 2002) found a significant upregulation in circulating serum HSP72 in severely traumatized patients as early as 30 minutes after injury. Increased circulating serum HSP72 has also been measured in patients after coronary artery bypass grafting (Dybdahl *et al* 2002; Dybdahl *et al* 2004). Importantly, circulating serum HSP72 has been suggested as a marker of myocardial damage, and reported to have a role in the inflammatory response after acute myocardial infarction (AMI) (Dybdahl *et al* 2005). Other conditions in which elevated levels of circulating serum HSP72 has been demonstrated are renal disease (Wright *et al* 2000), hypertension (Pockley *et al* 2002), atherosclerosis (Pockley *et al* 2003), aging (Terry *et al* 2004), and sickle cell disease (Adewoye *et al* 2005). However, in these conditions although necrosis is proposed as the mechanism of release, conclusive experimental data is still lacking.

A more conclusive study was designed in which *in situ* killing of tumour cells using suicide gene transfer to generate death by a non-apoptotic pathway was shown to be associated with high immunogenicity and induction of HSP (Melcher *et al* 1998). The most conclusive demonstration that necrosis accounts for HSP release is seen following infection with lytic viruses. In a study by Moehler and coworkers, it was demonstrated that parvovirus-mediated cell killing enhances tumour immunogenicity by HSP72 release and contributes to the anti-tumour effect of parvoviruses (Moehler *et al* 2005). Although these authors did not directly demonstrate that H1-induced cell killing and its associated HSP72 release promotes the loading and maturation of antigen presenting cells and by extension triggers tumour-specific immune responses, one can speculate that the release of HSP72 could facilitate priming of T cells specific for viral antigens.

Taken together, the passive release clearly is an important mechanism by which HSP72 is released into the circulation. However, is it the only mechanism? An additional mechanism for HSP release is now proposed as being of equal importance in the release of HSP72 into the circulation.

4. Active mechanisms

The active release hypothesis has been proposed as an additional mechanism to the passive release hypothesis. Three lines of evidence strongly support this hypothesis. First, as early as 1998 Pockley and coworkers demonstrated the presence of soluble HSP72 and antibodies against HSP72 in the peripheral circulation of normal individuals (Pockley *et al* 1998). Second, Guzhova and colleagues demonstrated that HSP72 is released by glia cells in the absence of necrotic cell death (Guzhova *et al* 2001). Third, and extremely compelling, psychological stress induced by exposure of a Sprague Dawley rat to a cat results in the release of HSP72 into the circulation (Fleshner *et al* 2004). In this study, a rat was put in a glass cage and a cat placed on top of the cage. This form of psychological stress induced a marked increase in circulating HSP72, as judged by the classical HSP72 sandwich ELISA. The terrified rat did not run around the cage thereby negating the possibility that damage to the muscles played a part in increased HSP72 release. Subsequent studies have demonstrated that HSP72 is released by B cells (Clayton *et al* 2005) and peripheral blood mononuclear cells (Hunter-Lavin *et al* 2004) under non necrotic conditions.

Using conditions that will not induce significant cell death, our group showed that IFN-γ and IL-10 induce the active release of constitutively expressed HSP70 as well as designed Hsc70 or HSP73 from tumours (Barreto *et al* 2003). However, these initial studies did not address the mechanism underlying HSP72 release. Recently, our group (Bausero *et al* 2005a; Gastpar *et al* 2005) and others (Lancaster and Febbraio 2005) have begun to elucidate the mechanism of active release of iHSP72 from viable cells. In our study, we demonstrated that certain pro-inflammatory cytokines, normally found in high concentrations within inflammatory foci including IFN-γ, IL-10 but not the anti-inflammatory cytokine TGF-β1, mediate the active release of HSP72. We further showed that whereas some eHSP72 could be found as free HSP72, a proportion of eHSP72 was released within exosomes (Bausero *et al* 2005a). Exosomes are internal vesicles of multi-vesicular bodies (MVB) released into the extracellular milieu upon fusion of MVB with the cell surface (Raposo *et al* 1996; Zitvogel *et al* 1998, 1999). In addition to containing HSP72 (Bausero *et al* 2005a; Gastpar *et al* 2005), exosomes are highly packed with immunostimulatory mediators including MHC class I and II (Raposo *et al* 1996; Zitvogel *et al* 1998, 1999) and costimulatory molecules (Escola *et al* 1998). Additionally, we demonstrated that HSP72 is released by a non classical protein transport pathway and that intact surface membrane lipid rafts are required for efficient stress-induced HSP72 release (Bausero *et al* 2005a; Gastpar *et al* 2005). These studies were recently confirmed in B cells (Clayton *et al* 2005). Recent studies by Lancaster and Febbraio (2005) demonstrate that exosomes provide the major pathway for secretory vesicular release of HSP72. However, using methyl-β-cyclodextrin (the cholesterol depleting agent) to disrupt lipid raft function, these authors were unable to confirm a role for lipid rafts in stress-induced HSP72 release from human peripheral blood mononuclear cells (PBMC) (Lancaster and Febbraio 2005). In order to address the cellular location of HSP72 after stress, a recent study demonstrated that newly synthesized HSP72 protein localizes within the Golgi region of HeLa cells and also concentrates on the surface of the plasma membrane and in the ruffled zone of migrating cells (Schneider *et al* 2002).

Taken together, these studies suggest that the active release hypothesis is an important mechanism by which HSP72 is released into the circulation. However, studies remain to be performed that conclusively demonstrate that T cell responses are primed by active release of HSP72 especially in the case of psychological stress or exercise.

5. Function of HSP70 on NK cells

HSP70 is highly expressed on the surface of certain tumour cells and its surface expression is absent in normal cells (Multhoff *et al* 1997). This unique surface expression of HSP70 may be utilized in the tumour-specific immune response and lysis of tumour cells mediated by non-MHC restricted natural killer cells (Multhoff *et al* 1999; Moser *et al* 2002; Multhoff 2002; Gross *et al* 2003a). Furthermore, a 14-mer peptide isolated from HSP70, termed TKD peptide stimulates the cytolytic and proliferative activity of NK cells similar to that of full length HSP70 (Multhoff *et al* 2001). HSP70-based immunotherapies have been extended to other infectious diseases such as malaria and HIV (Lehner and Anton 2002; Lehner and Shearer 2002; Wang *et al* 2002).

In comparison with immunocompetent cells, malignant tumour cells express high levels of surface bound HSP70 (Botzler *et al* 1996; Multhoff and Hightower 1996; Multhoff *et al* 1997; Botzler *et al* 1998b; Hantschel *et al* 2000). This HSP70 expression on tumours correlates with an increased sensitivity to natural killer (NK)-mediated cytolysis following cytokine stimulation (Botzler *et al* 1998a; Multhoff *et al* 1999, 2001). Recent studies have shown that the cytolytic activity of HSP70 can also be transduced by specific fragments of the HSP70 protein. Both the full-length HSP70 protein and the C-terminal domain of HSP70 stimulates the cytolytic activity of naive NK cells against HSP70-positive tumour target cells (Gross *et al* 2003a). In addition, tumour growth in mice with severe combined immunodeficiency was shown to be inhibited by HSP70-peptide-activated, CD94 positive natural killer cells (Moser *et al* 2002). Recent work from the Multhoff laboratory demonstrates

that a 14 amino acid sequence of the HSP70 protein, termed TKD (TKDNNLLGRFELSG, $aa_{450-463}$) is the extracellular recognition site for NK cells (Multhoff *et al* 2001). These authors demonstrate that granzyme B specifically binds to portions of the HSP70 expressed on the plasma surface of tumours but not normal cells (Gross *et al* 2003b). These observations unravel a hitherto unknown mechanism by which cytolytic effector cells eliminate HSP70 expressing tumours in a perforin-independent, granzyme B-dependent manner. These studies are in agreement with recent findings that immunization of the peptide binding C-terminal portion of HSP70 ($aa_{359-610}$) ($HSP70_{359-610}$) is responsible for stimulating Th1-polarizing cytokine (IL-12 and TNF-α), C-C chemokine release, and acts as an adjuvant (Wang *et al* 2002). Immunization of nonhuman primates with $HSP70_{359-610}$ induced the production of RANTES and IL-12, and acted as an adjuvant when loaded with CC5-peptide (Wang *et al* 2002), suggesting a possible alternative vaccine strategy for HIV infection (Lehner and Anton 2002; Lehner and Shearer 2002). An additional role of extracellular HSP70 as a chemoattractant for NK cells was recently demonstrated (Gastpar *et al* 2005).

6. Biological significance of released HSP72

Irrespective of whether HSP72 enters the circulation via an active or passive release mechanism, what is the role of eHSP72 in circulation? The danger theory postulates that immune activation involves danger/non-danger molecular recognition schemas and suggests that innate immune cells are activated by danger signals that are derived from stressed or damaged self-proteins (Matzinger, 1998; Gallucci and Matzinger 2001). It is now widely accepted that eHSP72 fit this criteria. The hypothesis is further reinforced by studies showing that circulating eHSP72 is increased and upregulated in diseased conditions including renal disease (Wright *et al* 2000), hypertension (Pockley *et al* 2002), atherosclerosis (Pockley *et al* 2003) and sickle cell disease (Adewoye *et al* 2005). However, intriguing questions still remain about the role of increased circulating eHSP72 during psychological stress like that demonstrated when a Sprague Dawley rat is exposed to a cat (Fleshner *et al* 2004) or the release eHSP72 into circulation by human brain in response to exercise (Lancaster *et al* 2004).

Is it possible that in these situations the circulating eHSP72 is priming the immune system to real or perceived danger? These are important questions, the answers to which will provide a keen insight into numerous psychological and pathophysiological conditions. However, extensive studies and concerted efforts that bring together HSP researchers from such disparate fields as immunology, molecular biology, cell biology, neurophysiology and psychology utilizing new breakthrough technologies including the recently deciphered human genome and proteomics are required before conclusive answers can be given.

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