Chapter 9

Discussion

Heat shock protein 72 is one of the fundamental molecular chaperones in the cellular compartment but many studies have demonstrated an equally key role in inter-cellular signalling and in interactions with the immune response (Multhoff et al. 1998; Zugel and Kaufmann 1999; Asea et al. 2000; Wells and Malkovsky 2000; Srivastava 2002; Millar et al. 2003). The present study aimed to focus attention on the different means by which Hsp72 can be presented to the extracellular environment and therefore can deliver different messages to the organism. This was done by studying the differential expression of Hsp72 in tissues in response to stress and by focusing on Hsp72 surface presentation and secretion mechanisms.

9.1 *In vivo* physiological models

The first model used was physiological stress in the form of exercise. The objective was to explore *in vivo* the possible sources of, and mechanisms behind, the increases in serum Hsp72 that are observed during intensive exercise (Fehrenbach et al. 2000; Walsh et al. 2001; Febbraio et al. 2002; Milne and Noble 2002; Fehrenbach et al. 2005). Tissue specific up-regulation of Hsp72 have been proposed from indirect measurements in humans (Asea et al. 2000; Febbraio et al. 2002; Febbraio et al. 2002; Millar et al. 2003; Lancaster et al. 2004). Previous mechanisms proposed include passive release into serum as a result of cellular damage (Basu et al. 2000; Saito et al. 2005) or direct secretion as a result of stimulation by pro-inflammatory cytokines or stress hormones (Barreto et al. 2000; Febbraio et al. 2002). In this study an animal model explored the tissue source of serum Hsp72 following intensive exercise and showed in line with literature (Febbraio et al. 2002; Febbraio et al. 2002; Febbraio et al. 2002; Lancaster et al. 2004) that the most stressed organs were liver and heart and to a lesser degree muscle, suggesting that they are potential sources of increased serum Hsp72 (Walsh et al. 2001; Fehrenbach et al. 2005). However the data cannot rule out brain or adrenal glands which express a lower level and could have a delayed response. Interestingly Hsp72 is up-regulated in muscle tissue which is the most affected by exercised, as shown with other models.
(Salo et al. 1991; Thompson et al. 2001; Febbraio et al. 2002; Febbraio et al. 2002; Febbraio et al. 2004; Suzuki et al. 2006). Hsp72 released from the cytosol but remained localized in the inter-myofibrillar space implying a localized protective role as opposed to a general systemic release into circulation. A proportion of this Hsp70 must be membrane bound as it co-localizes with the lipid raft marker GM-1. The heart and liver which are also affected by the exercise stress are not only able to express Hsp72, but also to release into the blood and therefore send to the whole body a moderate stress signal and stimulate an appropriate immune response. This is in line with other observations where Hsp72 in heart was found in proximity of blood vessels and where blood flow from liver showed high serum Hsp72 content (Kregel and Moseley 1996; Leger et al. 2000; Febbraio et al. 2002; Gonzalez and Manso 2004; Dybdahl et al. 2005; Starnes et al. 2005).

Intensity and duration of the exercise have both been proposed to be fundamental for the induction of increased serum Hsp72 (Fehrenbach et al. 2000; Walsh et al. 2001; Milne and Noble 2002; Whitham et al. 2004; Fehrenbach et al. 2005). In order to test the role of the intensity of the exercise regardless of the duration, an anaerobic workout, the “30 seconds maximal intensity Wingate test” (Hussain et al. 1996; Akimoto et al. 2000; Roberts et al. 2007) was performed and the potential pro-inflammatory cytokines induction was attenuated by the anti-inflammatory drugs, aspirin and ibuprofen. The exercise model showed that Hsp72 is down-regulated in monocytes immediately after the exercise while there is a concomitant trend of increase in the Hsp72 release. This is in line with previous findings which showed that serum Hsp72 increased after intensive exercise (Walsh et al. 2001). The monocytes, therefore, potentially contribute to the increase in serum Hsp72 despite their relatively low numbers in circulation.

Despite the slight induction of the \textit{Hsp72} gene expression, the exercise protocol did not induce the release of inflammatory cytokines such as IL-6, TNF-\(\alpha\), IL-10, demonstrating that anaerobic exercise is not as effective as prolonged aerobic exercise and thus the duration, more than intensity is important for the immune system activation, and Hsp72 signalling. Therefore, this data suggests that increases in serum Hsp72 induced by exercise can be independent from pro-inflammatory cytokines. Combining the data from the pig and human protocols, suggest that the intensity and
duration of exercise not only effect the degree by which serum Hsp72 increases, but also the source and mechanisms involved. It is well established that intracellular Hsp72 protects cells from insult and is an effective anti-apoptotic agent (Beere et al. 2000; Li et al. 2000; Saleh et al. 2000; Garrido et al. 2001; Nylandsted et al. 2004; Bivik et al. 2007). Extracellular Hsp72 can be taken up by a range of cells and it then provides protection to these cells (Plumier et al. 1996; Guzhova et al. 2001; Kustanova et al. 2006). It may well be that extracellular Hsp72 present in serum at low levels can provide protection to a range of cells and tissues in the body.

9.2 In vitro models of Hsp72 translocation

In order to test in vitro the molecular induction of Hsp72 following exercise, few events that occur during exercise were reproduced in vitro activated monocyte cell lines were used for the study in order to have a similar model to typical exercise protocols. The events that occur during exercise are release of pro-inflammatory cytokines and release from the adrenal gland of adrenormones epinephrine and norepinephrine which had been shown to stimulate increases in serum Hsp72 in vivo (Barreto et al. 2000; Febbraio et al. 2002; Fleschner et al. 2004; Bausero et al. 2005; Fleschner and Johnson 2005; Johnson and Fleschner 2006; Ortega et al. 2006). The pro-inflammatory cytokines IL-6 and IFN-\(\gamma\) up-regulate intracellular Hsp72 levels in monocytes and induce an active secretion as confirmed previously in different cell models (Figure 9.1) (Barreto et al. 2000; Bausero et al. 2005). The figure below summarises the effects of pro inflammatory cytokines on the Hsp72 cellular translocation and secretion.
IL-6 | IFN-γ | TNF-α

Hsp72 intracellular surface presentation release | Hsp72 intracellular surface presentation release | Hsp72 extracellular following necrosis

Figure 9.1: Pro-inflammatory cytokines treatment effect on Hsp72 cellular localization and secretion. Model of Hsp72 (red) movements in cells (blue circles represent viable cells, while blue broken circles represent necrotic cells) from the intracellular compartment into the plasma membrane (associated with the blue circle) and release outside the extracellular compartment.
While, contrasting to the literature (Johnson and Fleshner 2006), epinephrine and norepinephrine induce accumulation of Hsp72 inside the cells, but blocks or does not affect the surface presentation and release of Hsp72. However, co-treatment with the α1-adrenoreceptor blocker prazosin, which was thought to be the receptor involved in Hsp72 activation (Johnson and Fleshner 2006; Ortega et al. 2006), induces movement from the intracellular compartment to the cells membrane and induces the secretion (Figure 10.2), suggesting an additional role beyond the adrenoreceptor blocker prazosin, possibly an activator of another membrane transporter such as ABCG2 (Nishimura et al. 1986; Litman et al. 2000).

Figure 9.2 : Effect of Adreno hormones treatment and Hsp72 movement. Model of Hsp72 (red) cellular (blue circles) movement from the intracellular compartment to the plasma membrane (in association with blue circles) or with adrenoreceptors (dark blue structures on the membrane) or alternative membrane receptors (purple structure) to be identified more in detail.
The second model used in the study aimed to focus the attention on a system where Hsp72 was not up-regulated in response to an external stress but it was a consequence of the state of the cells, an event often found in diseased cells. Different blood malignancies, where Hsp72 had not been studied in detail before, were taken into consideration and Hsp72 localisation was screened in relation to the progression of the disease. Intracellular Hsp72 is present in other leukaemia types like acute myeloid leukaemia (AML) and is associated with a poor prognosis (Thomas et al. 2005). The cellular Hsp72 location seems to determine in many cancer types whether the protein is beneficial or detrimental to tumor cell survival (Sherman and Multhoff 2007), although a recent study suggested that elevated levels of Hsp72 in cancer cells, despite the location, is detrimental (Steiner et al. 2006). The screening of three different diseases types, CLL, MDS, AML, demonstrated an abnormally high Hsp72 intracellular and surface level in some patients. Hsp72 can be up-regulated both inside and on the surface of leucocytes from CLL, MDS and AML and can also be found in low, level in leucocytes from normal people. Patients can be divided into two distinct groups: the high-expressing Hsp72 or the low-expressing Hsp72 for both intracellular and surface levels. The intracellular level correlated with lymphocytes count and inversely correlated with time since diagnosis. Preliminary studies suggest that Hsp72 can be potentially manipulated through chemotherapy agents. This suggests that Hsp72 up-regulation can be an adaptation mechanism for cancer cells to the disease state and can be modified with the progression of the disease (Hwang et al. 2003). It was also interesting to note that Hsp72 can be present embedded on the surface of normal patients in a low but significant level. The seemingly contrasting roles of intracellular and surface Hsp72 (Mosser et al. 2000; Beere and Green 2001; Garrido et al. 2001; Garrido and Solary 2003; Gyrd-Hansen et al. 2004; Nylandsted et al. 2004) do not match the data on Hsp72 and prognosis (Steiner et al. 2006). Although it is clear that manipulation of Hsp72 location can be a successful therapeutic strategy (Krause et al. 2004; Milani et al. 2005).
The third model used in the study was the *in vitro* model aimed to explore the surface Hsp72 presentation and secretion. Hsp72 was modulated by different treatment that generate different degree of stress.

Normal blood cell analysis as negative control in the leukaemia screening showed surprisingly that even normal leukocytes can present Hsp72 embedded on the membrane, in apparent contrast with literature (Multhoff et al. 1995). This low expression can be modulated as in cell lines, but with a different degree of activation, by heat shock at 42°C where an up-regulation of the surface Hsp72 could be found, especially in the monocyte population. In other words even viable and healthy cells can sense the stress in the same way as abnormal cells do, but possibly to a smaller degree. The level of surface expression may determine responses to it. Heat shock stress modulates effectively surface Hsp72 presentation or secretion from cells: a sub-lethal heat shock at 42°C induces surface Hsp72 insertion in association with PS in the context of lipid rafts possibly as a response of the fluidisation of the membrane following heat shock (Arispe et al. 2002; Broquet et al. 2003; Arispe et al. 2004; Vigh et al. 2005; De Maio et al. 2007; Vigh et al. 2007; Vega et al. 2008). Hsp72 secretion increased at this temperature with time as the lysosome marker LAMP-1 appeared on the plasma membrane. The cells at this temperature are apoptotic since several apoptotic markers are induced: this implies that stressed cells can go through apoptosis but this process does not exclude surface Hsp72 presentation or secretion, despite previous literature postulated the contrary (Basu et al. 2000; Saito et al. 2005). A more severe stress resulted in a necrotic cell death. Under these conditions Hsp72 secretion increased and surface Hsp72 decreased (Figure 10.3). It also appears that at least two different pathways can operate for secretion and surface presentation. The first and more immediate is the PS - lipid raft associated surface Hsp72 presentation (Broquet et al. 2003; Arispe et al. 2004; Bausero et al. 2005; Wang et al. 2006) and a second that involves lysosomes, possibly a secretion through endo-lysosome vesicles as postulated previously (Mambula and Calderwood 2006). The Hsp72 release in this model, is comparable with surface insertion suggesting an increase of release on heat shock and a possible movement of the Hsp72 from the inside to the membrane (Figure 10.3). However one model that has been proposed
is that membrane insertion is part of the pathway leading to secretion (Mambula and Calderwood 2006).

Figure 9.3: Summary of the events occurring during heat shock time course and the effect on Hsp72 localization. Model of Hsp72 (red) translocation and cellular (blue circles, blue circle clusters and blue broken circles respectively represents healthy, apoptotic and necrotic cells) movement from the intracellular compartment, insertion into the plasma membrane through lipid rafts (orange structures on the membrane), lysosomal vesiculation (vesicle inside the cells at 3 and 4 hours) and release outside the extracellular compartment due to active release from healthy cells (1 hour), apoptotic cells (2, 3 and to a lesser degree 4 hours) and necrosis (4 hours).
Inhibitor data from caspase inhibitors, and the necrosis inhibitor, presented here support the previous suggestion that surface presentation and secretion can be separate processes. For example, inhibitors or Caspase 3 activation pathways result in increased Hsp72 secretion in the absence of Hsp72 insertion into the membrane, Hsp72 membrane insertion in the absence of secretion with the IM54 compound that block necrosis, and Hsp72 membrane insertion and secretion in the absence of PS externalisation by inhibition of the caspase-2 pathway. This demonstrates that Hsp72 secretion and surface insertion are not always controlled by the same mechanism. Membrane Hsp72 insertion can be regulated not only with PS/lipid rafts but could be associated with alternative structures, possibly lysosomes as showed before or ABC trasporters (Figure 10.4) (Mambula and Calderwood 2006).

![Figure 9.4: Apoptosis inhibitors and Necrosis inhibitor effects in the Hsp72 localization at normal growing temperatures (37°C) and heat shocked at 42°C and 45°C. Model of a normal cell left at 37°C or heat shocked at 42°C and 45°C in the centre of the figure, with Hsp72 (red) membrane insertion in lipid raft structures (orange structures in the membrane) and release in the extracellular environment. The four figures in the corners represent the effects of co-treatment with the apoptosis inhibitors used in the Hsp72 cellular localization.](image-url)
Because ABC transporters were considered as a candidate for Hsp72 membrane insertion and secretion, several inhibitors of different ABC transporters were used demonstrating that indeed Hsp72 movement is regulated by these transporters which seems to be essential for surface presentation and release and play different roles depending on the types: ABCA inhibition blocks surface presentation and release, ABCB cation channels blocks surface presentation but not release, ABCB calcium channels induce surface presentation and block release ABCC transporters block surface presentation but induce release. When ATPase activity of all the ABC transporters was inhibited both Hsp72 surface presentation and secretion, were blocked. Again the data suggests that there are more than one pathway to membrane insertion and secretion and that they can be independent processes (Figure 10.5). Although the study present concentrated on the inhibitory effect of ABC transporter blockers, the resulted discussed above cannot rule out that other secretion pathways, independent form ABC transporters, might be affected following treatments with these compounds. Each ABC transporter inhibitor induced Annexin V external exposure suggesting that a plasma membrane rearrangement and a modification could take place with a consequent internal protein secretion which could be independent form any membrane transporters.
Figure 9.5: ABC transporters inhibitors treatment at normal growing temperatures and at 42°C. Proposed model of Hsp72 (red) movement from the intracellular compartment to the external environment (outside the blue circle) through different ABC receptors in the presence or not of lipid rafts (orange structures in the plasma membranes). The first figure refers to inhibition of ABC transporters at normal growing temperature (37°C) while the second figure refers to the ABC transporters inhibition in heat shock conditions at 42°C. External Hsp72 represents a stimulatory effect on the Hsp72 secretion, while crosses on the transporters shows inhibitory effect on the Hsp72 secretion.
In general when Hsp72 is in association with PS in the plasma membrane it is more likely to be blocked in this compartment and not be secreted. However when Hsp72 is not associated with PS the Hsp72 movement to the cell surface seems to be transient and directed to the release outside the cells. This is confirmed by using membrane disrupting agents at low concentrations which induce heat shock factor activation (Basse et al. 1992; Balogh et al. 2005; Nagy et al. 2007) and membrane Hsp72 presentation (Botzler et al. 1996). Alcohol treatment demonstrated that their membrane disruption properties, not cytotoxic for the cells, induced a surface Hsp72 insertion PS-dependent and blocked Hsp72 release. PS presentation, together with increasing caspase-3 activation demonstrate that cells become apoptotic, suggesting that apoptotic cells derived from membrane fluidization express surface Hsp72 but don’t secrete the protein. The alkylating agent edelfosine at low concentrations impairs the external PS presentation (Zaremberg et al. 2005; Gajate and Mollinedo 2007; Ausili et al. 2008) without blocking the surface Hsp72 plasma membrane insertion even in apoptotic or necrotic conditions, demonstrating that surface Hsp72 presentation can occur independently from the association with PS and that it is likely to involve an additional system. High concentrations of edelfosine together with apoptotic and necrotic heat shock conditions, also block this alternative Hsp72 surface presentation suggesting that the compound, at high concentrations could impair other membrane structures involved in the surface Hsp72 presentations, one of which could be multidrug transporters (Perez-Victoria et al. 2001; Zaremberg et al. 2005; Gajate and Mollinedo 2007; van der Luit et al. 2007; Ausili et al. 2008) The data strongly suggest that additional membrane structures like ABC transporters are associated with the Hsp72 insertion in the membranes and secretion, which can be considered two independent processes (Figure 10.6).
Figure 9.6: Membrane interacting agents BA, PhA and Edelfosine and Hsp72 movement and secretion. Model of Hsp72 (red) plasma membrane presentation and secretion from cells (blue circles represents viable cells while blue circle clusters represents apoptotic cells) following BA, PhA and Edelfosine treatment. The surface presentation could be in association with lipid rafts (orange structures on the plasma membrane) or with the involvement of membrane transporters, possibly ABC transporters (green structures on the plasma membrane).
Hsp72 can deliver information locally, to neighbouring cells, or globally, to the whole organism, by its interaction with other cellular structures and depending what, if any, client protein is being chaperoned. Hsp72-peptide associations in cancer result in Hsp72 binding to immunocompetent cells through specific receptors and this then activates cell-mediated immunity, through MHC-I presentation and activation of cytotoxic T-lymphocytes with concomitant release of pro-inflammatory cytokines (Blachere et al. 1993; Basu et al. 2000; Binder et al. 2000; Sondermann et al. 2000; Binder et al. 2001; Srivastava 2002; Theriault et al. 2006). Membrane embedded Hsp72 presentation in certain cancer cells can also activate the immune system in a different way, by activation of natural killer cell activity and consequent apoptotic cell death of the target cells (Botzler et al. 1998; Multhoff et al. 1999; Gehrmann et al. 2003; Gross et al. 2003; Gross et al. 2003; Gross et al. 2003; Gastpar et al. 2005; Milani et al. 2005; Radons and Multhoff 2005). The amount of Hsp72 produced in the cells and the location that they assume determine the nature and strength of the signal delivered. High intracellular level of Hsp72 and surface presentation in certain cancer types is associated with the severity of the disease, or the responsiveness against chemotherapy drugs. High level of serum Hsp72 is associated with autoimmune diseases, and with the production of antibodies against the Hsp72 protein (Stephanou et al. 1998; Zugel and Kaufmann 1999; Pockley et al. 2002; Millar et al. 2003; Pockley et al. 2003; Kustanova et al. 2006; van Eden 2006). Physiological stress such as exercise can induce an Hsp72 up-regulation in different cellular compartments and can induce an extracellular Hsp72 in the blood circulation depending on the intensity and the duration of the stress (Fehrenbach et al. 2000; Walsh et al. 2001; Febbraio et al. 2002; Milne and Noble 2002; Febbraio et al. 2004; Gonzalez and Manso 2004; Lancaster et al. 2004; Fehrenbach et al. 2005; Starnes et al. 2005). Psychological stress and also age with its increased oxidative stress susceptibility can alter Hsp72 level either inside the cells or outside determining a regulation of the inflammation of the susceptibility to external pathogens (Njemini et al. 2003; Fleshner et al. 2004; Fleshner and Johnson 2005; Johnson and Fleshner 2006; Terry et al. 2006; Njemini et al. 2007; Njemini et al. 2008; Ogawa et al. 2008). Bacterial attack results in an up-regulation of Hsp72 release with activation of the immune response (Triantafilou et al. 2002; Dauphinee and
Karsan 2006; Triantafilou et al. 2008). In other words any attempt to modify cellular homeostasis by different types of stresses that are either external stress, like oxidative stress, bacteria, exercise stress, or psychological, or internal stresses like certain cancer types, or the ageing process, produce an Hsp72 response in the cell, either an up-regulation or down-regulation and a movement towards the cell surface or secretion to the extracellular environment.

Hsp72 is synthesised, translated and the protein product translocated in order to protect the cells from stress not only in its primary function as a chaperone, but also to deliver a “danger signal” to other cells. Hence Hsp72 can be certainly included as a molecule having an important role in the “danger signal” model. The original “danger hypothesis” proposed by Matzinger (Matzinger 2002) implied that the signal would be either exogenous like bacteria antigens or endogenous like typical intracellular molecules that are released into the extracellular milieu as a result of cell necrosis and tissue damage (Saito et al. 2005). The danger signal can act locally in order to produce a localised immune system response or distant from the release site when reaching the circulation in order to produce a more systemic immunological activation (Pockley et al. 1998; Campisi et al. 2003).

However a modification from this danger model has been presented recently from our team that postulates that the danger signal can be modulated in order to have an appropriate response (Williams 2007; Williams and Ireland 2008). The modulation can depend on the type of molecule used by the system, the amount of the protein expressed and the timing of the signal. The other important aspect regarding the new concept of danger signal is that it can also come from cells that are not necrotic, where cellular contents are released passively into the extracellular milieu. Evidence suggests that the danger signal, in particular Hsp72, can be delivered from live stressed cells that can actively secrete the protein through different release mechanisms, (Guzhova et al. 2001; Barreto et al. 2003; Broquet et al. 2003; Hunter-Lavin et al. 2004; Bausero et al. 2005; Tytell 2005; Evdonin et al. 2006; Mambula and Calderwood 2006; Chen et al. 2009) or can be delivered through apoptotic cells, despite earlier work suggesting otherwise (Basu et al. 2000).
This hypothesis is confirmed with the work presented here where stressed apoptotic cells present Hsp72 on the cell surface and release it in the external environment.

The model presented (Figure 10.7) can be amplified by saying that the danger signal message, Hsp72 in this case, is delivered to the target cells in states and concentration that depend on how the signal is delivered and hence on the state of the cell that produce Hsp72. Further the nature of the signal Hsp72 carries is determined by whether the cell presenting it is viable, stressed, apoptotic, necrotic, and the location of the Hsp72 in the cells. In fact under conditions of zero or low stress it may be considered a protective molecule and actually have immunosuppressive activity (Zugel and Kaufmann 1999; Leger et al. 2000; Tytell et al. 2000; Guzhova et al. 2001; Tytell 2005; Kustanova et al. 2006). In other words the secretion system used in the cells and the association with the membranes can be associated with deliver to the external environment a different degree of signal.
Low danger
- Release from normal cells
- Exercise stress localised in tissues with low serum Hsp72 levels
- Pro-inflammatory cytokines treatment
- Stress hormones treatment

Medium danger
- Apoptotic cells with membrane Hsp72, but no secretion
- Lipid raft mediated surface presentation
- Lipid raft mediated surface and secretion

Medium High danger
- Apoptotic cells with membrane and secreted Hsp72 through different release pathways
- Lipid raft mediated surface and secretion
- Lysosomes mediated surface and secretion
- ABC transporters mediated surface and secretion

High danger
- Necrotic cells

Figure 9.7: Proposed model of the significance of Hsp72 secretion and surface presentation. Hsp72 (red) membrane presentation in association with lipid rafts (orange structures on the plasma membrane) or ABC transporters (green structures on the plasma membrane) and secretion in viable cells (blue circles), apoptotic cells (blue circle clusters), necrotic cells (blue broken circles) or cells secreting lysosomes (blue circles with vesicles inside). The traffic light represents the different degree of danger level that Hsp72 can deliver to the whole organism: a green light is a low threat, a yellow light is an medium danger level and a red light is a high danger level for the organism.
Cellular Hsp72 location, the plasma membrane insertion and the differential release pathways which was found in this study in response to several stressors, can certainly be considered a different degree of strengths and type of danger signal.
Hence a revised danger model can be proposed:
A “Low danger” signal, where the cells were subjected to physiological stresses such exercise; in this case the cells which are more involved in the stress, are not damaged by the stress, but up-regulated Hsp72 and partially release in the local site as showed in muscle fibers from exercised subjects: this provides protection to local cells and tissues, and dampens undesired systemic inflammatory reactions (Lamb et al. 2003; Ghayour-Mobarhan et al. 2005; Ghayour-Mobarhan et al. 2005; Suzuki et al. 2006; Njemini et al. 2007; Njemini et al. 2007). The intensity but mainly the duration of the exercise as shown with the two exercise protocol used, determine a possible increase of the danger signal by increasing the level of circulating Hsp72 and the release from other organs (Milne and Noble 2002; Fehrenbach et al. 2005; Peake et al. 2005).
This is the same case for cytokines and adrenormones in-vitro stimulation where cells are stressed but are still healthy and present the surface Hsp72 and secrete at moderate levels(Febbraio et al. 2002; Barreto et al. 2003; Bausero et al. 2005). The “Medium Danger” occurs when the cells are suffering the stress and experience a blocking of proliferation and start to became apoptotic. This expresses different mechanisms:
• This is the case when membrane disrupting agents were used where apoptotic cells express surface Hsp72, but do not release the chaperone. Because it is needed in the membrane in order to stabilise the structure form the damages derived form the treatment (Vigh et al. 1998; Vigh et al. 2007)
• There is also the case where apoptotic cells express surface Hsp72 and secrete it following heat shock.
The medium danger signal, increased surface Hsp72 and the Hsp72 secretion from apoptotic cells is delivered through differential ways involving as demonstrated, lipid rafts (Broquet et al. 2003; Arispe et al. 2004; Bausero et al. 2005; Chen et al. 2005; Wang et al. 2006), lysosome vesicles and
different ABC transporters (Mambula and Calderwood 2006), although exosomes vesicles involvement can’t be excluded (Bausero et al. 2005; Clayton et al. 2005; Gastpar et al. 2005; Lancaster and Febbraio 2005). Modulation of the apoptotic cell death pathways did change the signal delivery type but did not block it, demonstrating that the cells evolved alternative signalling methods. Apoptotic cells expressing Hsp72 could be target for macrophages recognition and phagocytosis as previously demonstrated (Wang et al. 2006; Vega et al. 2008) and by data from the lab (H.E. Ireland & H Williams unpublished data), but also extracellular Hsp72 is a target for macrophages activation (Vega et al. 2008).

The “High danger” is the extreme signal and the one contemplated in the original “danger signal model” and occur in the case of severe stress that determine a cytotoxic effect with necrosis: this occur in the case of severe heat shock at 45°C when cells can’t recover from the stress and need to send a signal that would alert and strongly activate the immune system.

This differential Hsp72 movement and expression that is organ and stressor specific suggests that the cellular system developed as result of thousands years of evolution, to utilise multiple cell signalling pathways. The present study confirms that in response to different stimuli, cells present Hsp72 on the cell surface and secrete in different ways which are appropriate to the stimulus and cell type. There is therefore potential for Hsp72 to present different danger signals to the immune system depending on stressor type and intensity. This raises the potential for manipulation of the response in developing novel therapies against diseases such as cancer.