

# Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: A form of communication during injury, infection, and cell damage

It is never known how far a controversial finding will go!  
Dedicated to Ferruccio Ritossa

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**Abstract** Heat shock proteins (hsp) have been found to play a fundamental role in the recovery from multiple stress conditions and to offer protection from subsequent insults. The function of hsp during stress goes beyond their intracellular localization and chaperone role as they have been detected outside cells activating signaling pathways. Extracellular hsp are likely to act as indicators of the stress conditions, priming other cells, particularly of the immune system, to avoid the propagation of the insult. Some extracellular hsp, for instance Hsp70, are associated with export vesicles, displaying a robust activation of macrophages. We have coined the term Stress Observation System (SOS) for the mechanism for sensing extracellular hsp, which we propose is a form of cellular communication during stress conditions. An enigmatic and still poorly understood process is the mechanism for the release of hsp, which do not contain any consensus secretory signal. The export of hsp appears to be a very complex phenomenon encompassing different alternative pathways. Moreover, extracellular hsp may not come in a single flavor, but rather in a variety of physical conditions. This review addresses some of our current knowledge about the release and function of extracellular hsp, in particular those associated with vesicles.

**Keywords** Heat shock proteins · Stress · Exosomes · Microparticles · Cellular communication · Nonclassical secretory pathway · Inflammatory mediators

## Introduction

Under normal physiological conditions, cells interact with each other to synchronize their metabolic activity, gene expression, and other basic cellular processes. In other words, cells communicate to maintain homeostasis. When cells are exposed to environmental changes, such as a variation in the availability of nutrients, they communicate to adjust their metabolism for the new condition. The same situation is true when cells are confronted by stressful circumstances. Thus, stressed cells release signals to alert the rest of the organism of a potentially damaging situation. We have coined the term Stress Observation System (SOS) for the mechanism responsible for the sensing of stress conditions in the extracellular environment. Some of the stress signals released by cells correspond to heat shock proteins (hsp), which are expressed in response to the insult. The mechanism involved in the release of hsp is still very mysterious, and their role in cell activation is only just beginning to be understood. This article presents a perspective on the release and role of extracellular hsp, in particular those hsp associated with membrane vesicles, in the activation of the immune system to combat injury and infection.

Expression of hsp: the universal response to stress

Almost 50 years ago, the Italian scientist Ferruccio Ritossa discovered the heat shock response by serendipity when one of his coworkers changed the temperature of his cell incubator. Ritossa observed a novel change in the pattern of *Drosophila* salivary gland puffs. As a good scientist, who kept impeccable records, he related the changes in the puffs with the higher temperatures, repeated the experiments with the appropriate controls, and the heat shock response was

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born. However, his discovery was initially rejected because, in the words of a prominent journal editor, “it was irrelevant to the scientific community” (Ritossa 1996), a story too often repeated with many other novel observations that challenge the conventional wisdom. Later, the stress response was correlated with the expression of hsp. A large number of hsp have been identified so far, and they are classified according to their molecular weight into discrete families. As is the case in many other disciplines, several names have been given to the same hsp. A consensus nomenclature has recently been proposed to avoid this problem (Kampinga et al. 2009). Some hsp are present in normal nonstress conditions, playing important roles in different intracellular processes, among which their role as molecular chaperones is the most recognized. In addition, their expression is enhanced or induced after a variety of stresses, including environmental and pathological conditions. The presence of hsp is important for the recovery from stress and protection from subsequent insults (De Maio 1999; Giffard et al. 2008). The biology of intracellular hsp during normal and stress conditions has been summarized by a large number of reviews (Lindquist and Craig 1988; Morimoto 1991; Bukau et al. 2006; Hartl and Hayer-Hartl 2009). Consequently, this aspect will not be further discussed in this article.

A new twist in the stress field is the detection of hsp outside cells. Initially, a heat-shock-like protein was described as a glia-axon transfer protein of the squid giant axon (Tytell et al. 1986). Independently, Hightower and Guidon (1989) found that Hsp70 was released from cells by a mechanism that cannot be blocked by inhibitors of classical secretory pathways. This observation, like Ritossa’s discovery, was initially deemed irrelevant and impossible, in spite of a large number of appropriate controls. Thus, these early observations regarding the presence of hsp in the extracellular environment were disregarded for many years. It was not until another controversial finding was reported by Asea and Calderwood (Asea et al. 2000), who found that recombinant Hsp70 was capable of activating cells of the immune system, that a possible role for extracellular hsp was reborn. The results from Asea and Calderwood were disputed based on the possibility that the activation of immune cells was due to contamination by bacterial endotoxin (Gao and Tsan 2003; Bausinger et al. 2002), or other agents (Bendz et al. 2008). These worries have been ruled out by the use of recombinant Hsp70 isolated from insect cells, nonrecombinant Hsp70, treatment with polymyxin B, boiling, or incubation in serum-free medium (Srivastava 1997; Vega et al. 2008; Zheng et al. 2010). Today, it is well established that Hsp70 is, indeed, responsible for the activation of macrophages, monocytes, dendritic cells (DC), natural killer (NK) cells, and hepatocytes, independently of contaminants (Asea et al. 2000; 2002; Basu et al. 2001; Vabulas et al. 2002; Gastpar et al.

2004; Wang et al. 2006; Kovalchin et al. 2006; Aneja et al. 2006; Vega et al. 2008; Galloway et al. 2008). Moreover, extracellular hsp have been shown to act as potent immunostimulatory or immunosuppressive molecules depending on the circumstances by which they interact with cells (Pockley et al. 2008).

Another controversial finding was related to the origin of extracellular hsp. Although Hightower and Guidon (1989) demonstrated that the release of Hsp70 was from healthy cells, others proposed that the major source of extracellular Hsp70 was due to cell lysis after necrosis (Basu et al. 2000). Hunter-Lavin et al. (2004) reported that Hsp70 was released by an active mechanism independent of cell death, confirming Hightower and Guidon’s (1989) observations. However, these studies did not rule out the possibility that necrosis could be an additional source of extracellular Hsp70. In fact, we have observed that following regional hepatic ischemia/reperfusion injury in the rat, the majority of Hsp70 in circulation is due to liver necrosis (Vazquez and De Maio, unpublished observations). Consequently, there are two different sources of extracellular Hsp70: active, due to a nonconventional secretory process, and passive, secondary to cell death and lysis. In addition to Hsp70, other hsp have been detected outside cells, such as Hsp60 (Merendino et al. 2010), Hsp90 (Tsutsumi and Neckers 2007; Sidera and Patsavoudi 2008), Grp78 (Delpino and Castelli 2002; Kern et al. 2009), and Hsp27 (Liao et al. 2009). The importance of extracellular hsp has been enlightened by the detection of Hsp70 in the serum of patients suffering from an array of conditions (Table 1). In addition, hsp have been found in the serum of apparently healthy individuals (Pockley et al. 1998). The presence of Hsp70 in circulation has also been correlated with improved survival of critically ill patients (Pittet et al. 2002; Ziegler et al. 2005). Other extracellular hsp, including Hsp27 (Liao et al. 2009), Hsp60 (Zhang et al. 2008), and Hsp90 (Tsutsumi and Neckers 2007) have been additionally linked to several diseases.

#### Mechanisms for the export of hsp

The fact that hsp can be detected outside cells has been puzzling, since the majority of these proteins lack the consensus signal required for secretion via the classical endoplasmic reticulum (ER)-Golgi pathway. With the exception of ER-resident hsp (Grp78 and Grp94), the majority of hsp are synthesized in the cytosol and exported to the extracellular space by an active mechanism that cannot be blocked by typical inhibitors of the ER-Golgi pathway, such as brefeldin A. Consequently, they are likely to be released by an alternative mechanism, coined the nonclassical secretory or unconventional pathway (reviewed by Nickel and Sedorf 2008). It appears that

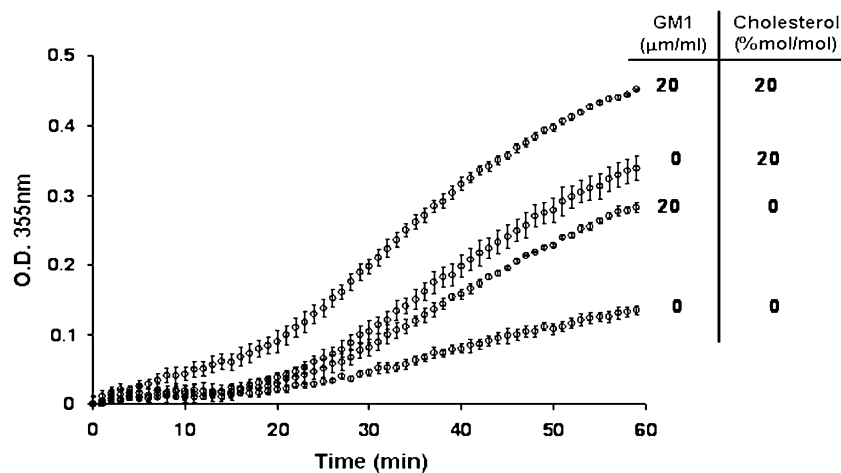
**Table 1** Extracellular Hsp70 in disease

Disease	Reference
Acute lung injury	Ganter et al. 2006
Cancer	Azuma et al. 2003; Faure et al. 2004
Chronic inflammation during aging	Njemini et al. 2004
Coronary artery disease	Zhu et al. 2003; Genth-Zotz et al. 2004; Zhang et al. 2010a
Diabetes	Oglesbee et al. 2005
Hypertension/pregnancy	Molvarec et al. 2006
Infection	Njemini et al. 2003
Ischemia/reperfusion	Hecker and McGarvey 2010
Myocardial infarction	Dybdahl et al. 2005
Trauma	Pittet et al. 2002; Ziegler et al. 2005

the nonclassical secretory pathway is not a unique mechanism, but rather a collection of alternative pathways, which have as a common denominator the exclusion from the ER/Golgi compartment. The most prominent molecules secreted by the unconventional pathway are interleukin (IL)-1 $\alpha$  and -1 $\beta$ , which are apparently released by several possible mechanisms (Eder 2008). Similarly to IL-1, the release of Hsp70 outside cells has been reported via different mechanisms. A lysosome–endosome pathway has been proposed (Mambula and Calderwood 2006). In this process, Hsp70 is translocated into lysosomes, where the protein is spared from degradation and transported to the exterior of the cell via the endocytic process. Indeed, the presence of Hsp70 in the lumen of lysosomes has been reported previously (Nylandsted et al. 2004). The translocation of Hsp70 into lysosomes has been proposed via an ATP-binding cassette (ABC) transport-like system (Mambula and Calderwood 2006). Hsp70 has also been proposed to be released by secretory-like granules (Evdonin et al. 2006). We have argued that there is another mechanism for the export of Hsp70, which is mediated by the insertion of the protein into the membrane of export vesicles (Vega et al. 2008).

Another surprising and controversial finding has been the interaction of Hsp70 with membranes. Initially, Alder et al. (1990) detected currents after the addition of Hsp70 to unilamellar lipid vesicles. Ten years later, we showed that Hsc70 was capable of integrating into an artificial lipid bilayer, opening cationic conductance channels, which were very stable and regulated by adenosine triphosphate/diphosphate (ATP/ADP) (Arispe and De Maio 2000). Similarly, Hsp70 was also found to open ion conductance pathways in artificial lipid bilayers (Vega et al. 2008). These observations have been expanded by other studies in which both Hsc70 and Hsp70 were capable of inducing liposome aggregation in a time-, concentration-, and nucleotide-dependent manner (Arispe et al. 2002). Moreover, it was found that the specificity of Hsp70 and Hsc70 for lipids was highly related to the presence of phosphati-

dylserine (PS) within membranes (Arispe et al. 2004; Schilling et al. 2009). New observations also revealed that Hsp70-induced PS-liposome aggregation could be enhanced by the coaddition of monosialotetrahexosylganglioside (GM1) and cholesterol (Fig. 1). Other sphingolipids, such as globotriaosylceramide, have also been reported to enhance Hsp70 insertion into membranes (Gehrmann et al. 2008; Sugawara et al. 2009). Recently, the specificity of Hsp70 for other lipids, such as anionic phospholipid bis(monoacylglycero)phosphate, has been reported (Nylandsted et al. 2004). In contrast, liposomes made of phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol could not sustain Hsp70 membrane insertion and liposome aggregation, confirming that the presence of PS is fundamental for Hsp70 insertion (Arispe et al. 2004). Although both Hsc70 and Hsp70 channels showed the same specificity, their conductance was not identical (Arispe and De Maio 2000; Vega et al. 2008). Likewise, the kinetics of Hsc70- or Hsp70-induced liposome aggregation were different (Arispe et al. 2002), suggesting that these two proteins are, indeed, functionally different, at least in their interaction with membranes. The stability observed in Hsp70/Hsc70 channels is remarkable for a protein that does not contain any consensus hydrophobic domain that can explain membrane insertion. In other words, the incorporation of Hsp70/Hsc70 into membranes cannot be predicted on the basis of their amino acid sequences. The mechanism for the insertion of Hsp70 within the lipid membrane is not known, but it is likely dependent on membrane fluidity (Horvath et al. 2008). We have proposed that Hsp70 membrane insertion may be related to the flipping/flopping of PS across the lipid bilayer. It is also possible that Hsp70/Hsc70 oligomerizes prior to or during membrane insertion, which is likely necessary for ion channel formation. Hsc70 has been shown to form low-order oligomers (Schlossman et al. 1984; Gao et al. 1996; Fouchaq et al. 1999). The C terminus end of Hsp70/Hsc70 displays a  $\beta$ -sheet structure, which may act as an oligomerization center. Indeed, other



**Fig. 1** Liposome aggregation induced by Hsp70, effect of cholesterol and GM1. The aggregation assay was performed with PS liposomes prepared with or without GM1 (20  $\mu\text{g}/\text{ml}$ ), cholesterol (20% mol/mol) or a combination of both. Liposomes were incubated with Hsp70

(10  $\mu\text{g}/\text{ml}$ ) in 40 mM histidine-HCl, pH 6, 300 mM sucrose, 0.5 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$  at 25°C, and the change in absorbance produced as a result of the aggregation process was measured at 350 nm in a Hewlett Packard spectrophotometer, with data collected every 30 s

proteins with similar  $\beta$ -sheet structures, such as annexin, amyloid, and amylin, have been shown to form ion channels in artificial lipid bilayers (Rojas et al. 1992; Arispe et al. 1993, 1996).

The discovery of Hsp70 insertion into membranes provided an explanation for another divisive topic, the presence of hsp on the cell surface. Initially, Ferrarini et al. (1992) detected Hsp90 and, to a lesser extent, Hsp70 on the surface of several tumor cell lines. This observation was followed by a more extensive characterization of Hsp70 on the surface of transformed cells by Multhoff et al. (1995). These discoveries remained controversial for many years despite a large number of publications confirming this phenomenon (reviewed by Multhoff and Hightower 1996; Multhoff 2007). Several lines of evidence suggested that Hsp70 was not simply associated to a membrane protein, but rather inserted into the plasma membrane. The strongest argument for this observation was that only an antibody that recognized a small epitope on the C terminus end of the molecule was capable of detecting Hsp70 on the cell surface (Botzler et al. 1998). In addition, Hsp70 within the plasma membrane was found resistant to acid or basic washes (Vega et al. 2008; Gehrman et al. 2008). Interestingly, the protein within the cell surface was found resistant to solubilization by nonionic detergents (Vega et al. 2008), suggesting that the protein may be localized within detergent-resistant microdomains (DRM), or lipid rafts, which are rich in cholesterol and sphingolipids (Brown and London 2000). Indeed, Hsp70 has been detected within the DRM fraction isolated from cells (Triantafilou et al. 2002; Broquet et al. 2003; Hunter-Lavin et al. 2004; Chen et al. 2005; Wang et al. 2006; Vega et al. 2008). These observations are supported by our findings demonstrating

an increase in aggregation by addition of cholesterol and/or GM1 to PS liposomes (Fig. 1). The presence of Hsp70 and other hsp on the cell surface has been widely reported by many investigators under different physiological or pathological conditions (Table 2). Moreover, the observation that Hsp70 can be inserted into the plasma membrane opens the possibility that this association may be the gateway for the release of the protein to the extracellular space. In fact, Hsp70-containing vesicles, which were derived from the plasma membrane, have been detected in the extracellular medium (Gastpar et al. 2005; Vega et al. 2008; Chalmin et al. 2010). Hsp70 was found on the surface of these vesicles, presenting many of the characteristics of the protein within the plasma membrane, such as insolubility by Triton X-100 (Vega et al. 2008).

Another alternative mechanism for the export of hsp proteins may be related to the release of cargo within extracellular vesicles. Indeed, several hsp have been detected within extracellular vesicles (Table 3). It is very likely that cytosolic proteins, including hsp, are trapped or actively transported into the lumen of extracellular vesicles and are consequently released into the outer cellular space. Alterations in the vesicle integrity may result in the release of the cargo into circulation. A potential example for this mechanism is related to the release of high-mobility group box 1 (HMGB-1), which is not secreted by the ER-Golgi pathway (Gardella et al. 2002). HMGB-1 has been reported in extracellular vesicles derived from stimulated human epithelial cells (Liu et al. 2006). Thus, it is possible that HMGB-1 is packed into extracellular vesicles that are released into circulation, which eventually release their cargo, including this important inflammatory agent (Yang et al. 2005). This possibility matches the initial observation about the detection

**Table 2** Detection of hsp on the cell surface

Hsp	Suggested name	Cells	Remarks	References
Hsp70	HSPA	Human Ewing's sarcoma and osteosarcoma cells Human lung (LX-1) and mammary (MX-1) carcinoma cells Human oral dysplasia and squamous cell carcinoma Tumor biopsies Melanoma biopsies Acute myeloid leukemia cells Head-and-neck tumors Human neutrophils Human colon (CX2) and pancreas (Colo357) carcinoma cells Human HepG2 cells Human fibroblast-like synovial cells Colon carcinoma cells (CX2) Murine LL/2 lung carcinoma Human Leukemia cells (U-937)	HS/recovery          LPS       HS/recovery  Hypoxia  Heat shock	Multhoff et al. 1995 Botzler et al. 1998 Kaur et al. 1998 Hantschel et al. 2000 Farkas et al. 2003 Gehrmann et al. 2003 Kleinjung et al. 2003 Hirsh et al. 2006 Gehrmann et al. 2008 Vega et al. 2008 Sedlackova et al. 2009 Schilling et al. 2009 Tani et al. 2009 Lasunskiaia et al. 2010
Hsp70B	HSPA6	Human colon cells (HT-29, CRL-1809)	Proteasome inhibitors	Noonan et al. 2008
Hsp70/Hsp25	HSPA/HSPB1	Human mammary adenocarcinoma cells (4T1)	Normal/heat shock	Bausero et al. 2004
Hsp70/Hsp90	HSPA/HSPC	Human tumor cell lines GLC1 (microcitoma), lung carcinoma (P71 and A549), melanoma (MEL10 and M14), hepatoma (HEPA1)		Ferrarini et al. 1992
Hsp90	HSPC	Human neuroblastoma (NB69) cells		Cid et al. 2009; Tsutsumi and Neckers 2007
Hsp90 $\beta$	HSPC3	Multipotential mesenchymal precursor cells		Gronthos et al. 1999
Grp94	HSPC4	Meth A sarcoma  Xenopus lymphoid tumor cell lines	Enhanced HS and exposure to reducing agents	Altmeyer et al. 1996. Robert et al. 1999
Hsp60	HSPD1	Liver/spleen	Infection	Belles et al. 1999
Grp78	HSPA5	Rat exocrine pancreas Atherosclerotic lesion (293T endothelial cells)  Prostate cancer cells (DU 145)	ER stress  overexpression	Takemoto et al. 1992 Zhang et al. 2010b Liu et al. 2003 Arap et al. 2004
Hsp90/Hsp70/Hsp27	HSPC/HSPA/HSPB1	Dog neutrophils	H2O2	Camins et al. 1999
Hsc70	HSPA8	Bile duct formation in newborn rat liver	Morphogenic maturation	Mills et al. 2010

of HMBG-1 in circulation during late sepsis (Wang et al. 1999). Similarly, exocytosis of endolysosome-related vesicles has also been proposed for the release of IL-1 $\beta$  (Andrei et al. 1999; MacKenzie et al. 2001).

#### Cell communication via extracellular vesicles

There are several ways that cells can communicate with each other. The most common is via soluble molecules that are

placed in the extracellular environment and interact with adjacent or distant cells via specific receptors. The typical examples of this type of communication are hormones and cytokines. Cells that are placed together can also communicate via surface contact molecules, such as adhesion proteins, which have been coined “cellular synapses” (Ahmed and Xiang 2010). Moreover, cells in close proximity can exchange surface molecules by the direct transfer of membrane portions, which is known as trogocytosis, or by

**Table 3** Hsp detected within extracellular vesicles

Hsp	Cells	Reference
Hsc70/Hsp70	Reticulocytes	Mathew et al. 1995
Hsp90, Hsc70	Mesothelioma	Hegmans et al. 2004
Hsp70	PBMC	Lancaster and Febbraio 2005
Hsp70, Hsc70, hsp 27, Hsp 90	B cells	Clayton et al. 2005
Hsp70	DC	Thery et al. 1999
Hsp70	Colo357/CX2	Gastpar et al. 2005
Hsp70, Hsp90, grp78	Rat hepatocytes	Conde-Vancells et al. 2008
Hsp90	Dendritic cells (DC)	Chaput et al. 2006
Mortalin/Grp75	K562	Pilzer and Fishelson 2005
Hsp70	HepG2	Vega et al. 2008
Hsp70	EL4 thymoma, TS/A mammary carcinoma, and CT26 colon carcinoma	Chalmin et al. 2010
Hsp70	mycobacteria-infected ( <i>M. smegmatis</i> and <i>M. avium</i> ) RAW 264.7	Anand et al. 1999
Hsp90	A172, HT-1080, MDA-MB231	McCready et al. 2010

membrane tethers or nanotubes (Davis 2007). In addition, adjacent cells can exchange low-molecular-weight metabolites by passing them from cell to cell via gap junctions (De Maio et al. 2002). An alternative mechanism for cellular communication could be mediated by membrane vesicles released by one cell and captured by another. These vesicles contain surface molecules, lipids, and cargo, which can be of different natures, including proteins, nucleic acids, carbohydrates, and small molecules.

The importance of cellular communication via vesicles is that they contain a large number of molecules in a small volume. Thus, every target cell can be hit at the same time by a great number of recognition or signaling molecules, which is likely to be more effective than the activity of individual components in solution. For example, activation of macrophages by Hsp70 is increasingly elevated when the protein is associated with vesicles, more than 250-fold higher than the same concentration of the hsp in solution (Vega et al. 2008). This robust effect is likely due to the high concentration of Hsp70 within the vesicle. In fact, the concentration of a ligand within a 100-nm vesicle is theoretically calculated in the millimolar range, which is much larger than the circulating concentration of any hormone or other cellular activator agent. Moreover, the multiplicity of different molecules per vesicle adds a synergistic effect for the information transmitted to the target cell. In other words, different components of the vesicle could simultaneously activate various cellular pathways. The second advantage of communication via vesicles is that they can travel long distances before breaking down and releasing their cargo, which, indeed, protects the lumen components from factors that may affect their stability. The final stage for this mechanism of communication requires that extracellular vesicles are specifically recognized by

target cells. Thus, it could be postulated that these vesicles interact with specific cells via surface molecules or receptors. In other words, surface molecules on the vesicles could act as recognition signals (zip codes) that direct the vesicles to specific receptors on cellular targets. These surface molecules could be directly involved in the activation of cells, or this role could be mediated by other components within the vesicles, such as cargo molecules. The process of vesicle interaction with the target cells may require endocytosis or membrane fusion.

#### Extracellular vesicles have different origins

The presence of extracellular vesicles has been known for a long time. However, the terminology, as in any emerging field, has been confusing, hindering the understanding of the role and origin of these vesicles. Extracellular vesicles have been coined exosomes, ectosomes, and microparticles, among many other names. Exosomes are small export vesicles initially derived from the plasma membrane, but by a mechanism involving endocytosis (Thery et al. 2009). In contrast, microparticles and ectosomes are derived directly from the plasma membrane by the formation of membrane protuberances. Initially, microparticles were described as the “dust” released by platelets (Wolf 1967). Since it was not initially clear whether or not they contained a membrane component, microparticles was an appropriate term. Today, it is known that they are encapsulated by a membrane, derived from the plasma membrane, and they may be very large, reaching diameters of up to 1,000 nm (Hugel et al. 2005; Meziani et al. 2008). Consequently, they are not properly microparticles, but rather macrovesicles. In contrast, ectosomes, which are derived from the plasma membrane, are smaller, about 50–100 nm in diameter

(They et al. 2009). The composition of exosomes, ectosomes, and microparticles is likely to be different. Density in sucrose gradients has been used as a criterion to differentiate them. However, it is unclear whether exosomes and ectosomes indeed have different density. Since the common approach to isolate extracellular vesicles is by differential centrifugation, it has been very difficult to physically separate the various types of vesicles, making it complicated to determine their specific components or markers.

Exosomes were first described as being derived during the differentiation of reticulocytes as part of a mechanism to release unwanted molecules (Harding et al. 1983; Pan et al. 1985). They are derived by a process in which the plasma membrane is internalized by endocytosis, followed by the formation of late endosomes. In this last compartment, the membrane of the endocytic vesicle is invaginated inward and sealed, resulting in vesicles within larger vesicles, called multivesicular bodies (MVB). Then, MVB fuse with the plasma membrane, liberating the internal vesicles (exosomes), 40–100 nm, which are then free in the extracellular environment (Johnstone 2006; They et al. 2009). The major characteristic of this process is that the vesicles that are released have the same topology as the plasma membrane. However, not all plasma membrane components are present in exosomes, suggesting a level of specificity for their assembly. Exosomes have been found to contain transferrin receptor, tetraspanins (CD9, CD63, CD81, and CD82), and Glycosylphosphatidylinositol (GPI) anchored glycoproteins, such as acetylcholinesterase. In addition, exosomes are also rich in cholesterol and sphingolipids (Simpson et al. 2008; They et al. 2009). These observations suggest that DRM or lipid rafts are present in these extracellular vesicles (de Gassart et al. 2003). Exosomes also contain some members of the endocytosis pathway, such as Rabs, which have been implicated in exosome formation (Savina et al. 2002; Ostrowski et al. 2010). Moreover, the release of exosomes is apparently calcium-dependent (Savina et al. 2003). Since exosomes are made by invaginations of the endosomal membrane, they pinch out some cytosolic molecules. In fact, a protein that has been detected in many exosome preparations is Hsc70. Originally, it was speculated that Hsc70 was present because of its high cytosolic abundance (approximately  $10^7$  molecules/cell). However, other proteins present in large concentrations in the cytosol have not been detected in exosomes. Thus, the theory is that the composition of exosome cargo is also very specific. Exosomes have been observed derived from a large number of cells in different physiological conditions (Simpson et al. 2008), including B cells (Raposo et al. 1996), T cells (Blanchard et al. 2002), DC (Zitvogel et al. 1998), macrophages (Bhatnagar et al. 2007), and mast cells (Raposo et al.

1997). They have been also associated with an important role in cancer and pathogen biology (Schorey and Bhatnagar 2008; O'Neill and Quah 2008).

In contrast to exosomes, microparticles, or ectosomes, are derived directly from the plasma membrane without the involvement of the endocytic pathway. The mechanism implies the formation of membrane protuberances or blebs, which at critical sizes are sealed and released (Hugel et al. 2005; Meziani et al. 2008). These vesicles are also likely to contain plasma membrane components and several cytosolic factors that are imported into the emerging vesicle. Interestingly, ectosomes and microparticles are also very rich in lipid raft components (Hugel et al. 2005; Lopez et al. 2005). In fact, it has been proposed that microparticles are derived from lipid rafts (del Conde et al. 2005). Similarly with exosomes, microparticles, or ectosomes, are not absolute replicas of the plasma membrane, suggesting a level of selectivity in their formation. These vesicles also likely require actin polymerization for their formation. Consequently, it is possible that F-actin is present within them. As mentioned above, microparticles tend to be large (100–1000 nm) and can be isolated by low *g* force and characterized by flow cytometry (They et al. 2009; Gelderman and Simak 2008). Microparticles have been shown to display immunosuppressive function (Sadallah et al. 2008), and they have also been implicated in angiogenesis (Mostefai et al. 2008). Microparticles have been proposed as markers of endothelium dysfunction (Horstman et al. 2004) and other conditions (Smalley and Ley 2008; Meziani et al. 2008). Small vesicles, which are commonly called ectosomes, might also be released through budding of the plasma membrane. Thus, size is not the main criteria to classify extracellular vesicles as exosomes or ectosomes. Therefore, the term exosomes should be restricted to vesicles that are derived via MVB. In other words, not all 5- to 100-nm vesicles detected outside cells are exosomes. Microparticles, ectosomes, and exosomes are different than vesicles derived from apoptotic cells. During cell death, there is a significant bleed of the plasma membrane and a release of vesicles, which have been coined apoptotic blebs or apoptotic vesicles. These vesicles are likely part of the cell shrinking process during apoptosis (Hristov et al. 2004). Apoptotic blebs probably contain a large variety of cellular components (membrane, cytosolic, and nuclear) without any specificity. They are possibly of different sizes and denser than exosomes (They et al. 2009). Their biological role, if any, remains to be established.

#### Extracellular vesicles and the SOS

Cells also secrete extracellular or export vesicles in response to stress, which could be specifically coined

Stress Cellular Vesicles. The idea is that these extracellular vesicles contain particular molecules, such as hsp, whose expression is induced during the stress. These extracellular vesicles are recognized by other cell types, in particular cells of the immune system, as part of an assessment of the stress conditions. Particular components of extracellular vesicles may act as signals to activate a preemptive response in distant cells. We have coined this phenomenon the SOS. It is likely that the specific cellular response depends on the composition of extracellular vesicles. The presence of extracellular vesicles has been observed in a large number of disease conditions (Table 4). For example, extracellular vesicles that were isolated from the plasma of septic patients induced myocardial dysfunction in isolated rabbit hearts and rat papillary muscle preparations (Azevedo et al. 2007). The incidence of aortic valve stenosis has been associated with the presence of extracellular vesicles derived from platelets after high shear stress. These extracellular vesicles could further activate leukocytes and monocytes to produce more vesicles, resulting in endothelial cell injury (Diehl et al. 2008; Ahn et al. 2008). Extracellular vesicles

derived from macrophages infected with intracellular pathogens were found to activate uninfected macrophages by a Toll-like receptor (Tlr) and myeloid differentiation factor 88 (My88) mechanism. They also induced polymorphonuclear leukocyte recruitment in lungs after intranasal delivery (Bhatnagar et al. 2007). Export vesicles isolated in the bronchoalveolar fluid of allergen-tolerized mice were reported to prevent an allergic reaction (Prado et al. 2008). Extracellular vesicles have been purified from different sources, including epididymal fluid and seminal plasma (Gatti et al. 2005), bronchoalveolar fluid (Admyre et al. 2003), amniotic fluid (Keller et al. 2007), blood (Caby et al. 2005), and urine (Keller et al. 2007).

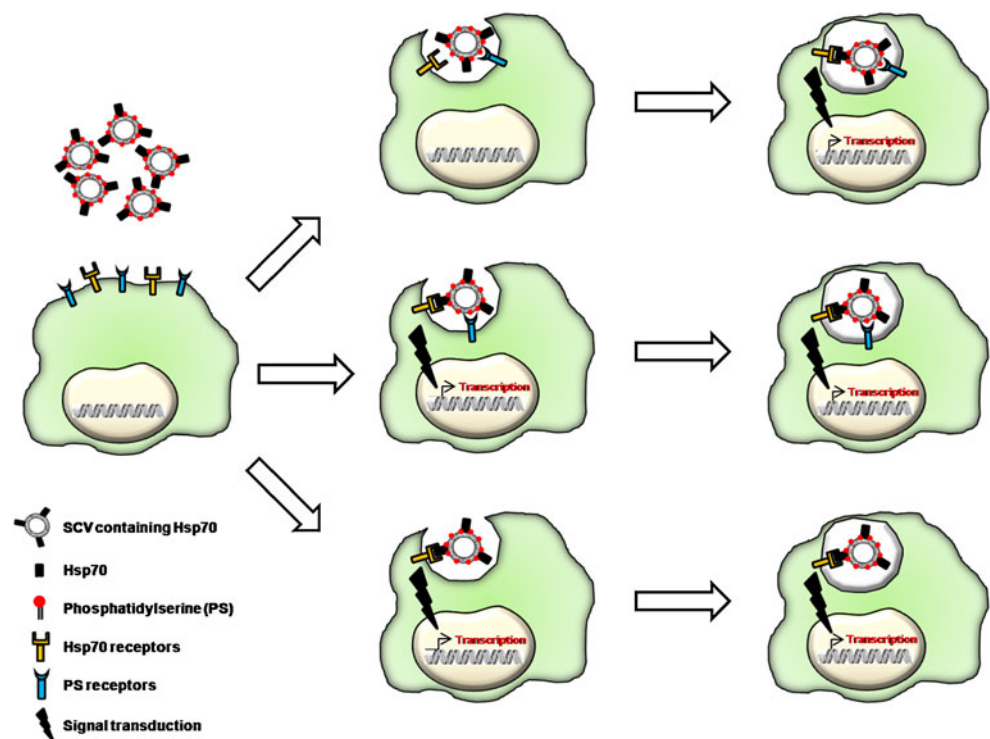
Hsp have been detected in preparations of extracellular vesicles from different cell types as mentioned before (Table 3). Moreover, these hsp-containing vesicles have been found to have a biological function. For example, vesicles containing Hsp70 on their surface displayed a robust and specific activation of macrophages, which was more robust than the same concentration of recombinant Hsp70 in solution (Vega et al. 2008). Hsp70-positive extracellular

**Table 4** Extracellular vesicles in disease

Disease	Source of ECV	References (101–125)
Acute Coronary Syndromes	Endothelial cells, platelets	Mallat et al. 2000; Bernal-Mizrachi et al. 2004
Acute ischemic stroke	Endothelial cells	Simak et al. 2006
Allergy	Bronchial alveolar fluids	Prado et al. 2008
Aortic valve stenosis	Platelets	Diehl et al. 2008
Arteriosclerosis obliterans	Platelets	Nomura et al. 2000
Cancer	Several tumors or cell lines	Hegmans et al. 2004; Gastpar et al. 2005; Chalmin et al. 2010
Cardiopulmonary bypass	Multiple sources	Nieuwland et al. 1997
Congestive heart failure	Endothelial cells	Rossig et al. 2000
Diabetes	Total, platelets, monocytes, endothelial	Koga et al. 2005; Sabatier et al. 2002; Nomura et al. 1995; Ogata et al. 2006; Leroyer et al. 2008; Esposito et al. 2008
End-stage renal disease	Endothelial cells	Faure et al. 2006
Erectile dysfunction	Endothelial cells	Esposito et al. 2008
Hypertension	Endothelial, monocytes, platelets	Preston et al. 2003
Infection	Macrophages	Bhatnagar et al. 2007
Lupus anticoagulant	Endothelial cells	Combes et al. 1999
Metabolic syndrome	Endothelial cells, tissue factor (+)	Diamant et al. 2002; Arteaga et al. 2006
Paroxysmal nocturnal hemoglobinuria	Platelets, endothelial cells	Hugel et al. 1999; Simak et al. 2004
Preeclampsia	Leukocytes	Bretelle et al. 2003; Gonzalez-Quintero et al. 2004
Pulmonary and venous embolism	Platelets, endothelial cells	Chirinos et al. 2005; Inami et al. 2003
Pulmonary hypertension	Endothelial cells	Amabile et al. 2008
Sepsis	Leukocytes	Nieuwland et al. 2000; Joop et al. 2001
Sepsis	Plasma	Azevedo et al. 2007
Sickle cell disease	Multiple sources	Shet et al. 2003
Thrombotic thrombocytopenic purpura	Platelets, endothelial cells	Kelton et al. 1992; Jimenez et al. 2003



**Fig. 2** Possible mechanisms for the interaction of extracellular vesicles containing Hsp70 and macrophages



vesicles were also found to stimulate the cytotoxic capacity of NK cells (Gastpar et al. 2005). Vesicles containing Hsp70 isolated from mycobacteria-infected cells induced an inflammatory response in macrophages (O'Neill and Quah 2008). In contrast, Hsp70-membrane-associated vesicles could induce an immunosuppressive effect (Chalmin et al. 2010). Thus, it is possible that Hsp70 within export vesicles plays different roles depending on composition, vesicle source, and cell target. The mechanism for the recognition of extracellular (stress) vesicles by target cells is not known. However, it is likely that they are specifically detected by the presence of surface signals. One possibility is that extracellular vesicles lose the lipid asymmetry typical of the plasma membrane (Zwaal et al. 2005), exposing PS moieties on the surface of the vesicle. Macrophages may engulf these vesicles via surface receptors that recognize this lipid, such as Tim-4 (Miyanishi et al. 2007) and BAI1 (Park et al. 2007). These receptors have been investigated in the context of uptake of apoptotic cells, which display PS on the surface as part of the cell death process. If extracellular vesicles are recognized by the presence of PS, it is likely that other components within the vesicle may be responsible for a secondary effect, such as the presence of Hsp70 (Fig. 2). Whether this effect occurs concomitantly via a surface receptor or during an endocytic process remains to be established. The other possibility is that molecules on the surface of extracellular vesicles are specifically recognized by receptors on the target cell (Fig. 2). For example, several receptors have been proposed to recognize Hsp70, including

Tlr 2 and 4 (Asea et al. 2002), CD14 (Asea et al. 2000), CD91 (Basu et al. 2001), CD40 (Becker et al. 2002; Wang et al. 2001), and scavenger receptors (Theriault et al. 2006; Facciponte et al. 2007). However, some of these observations have been controversial (Binder 2009). Whether these molecules are involved in the recognition of Hsp70-positive vesicles remains to be established. It could also be speculated that the presence of different stress signals of the vesicles may be recognized by various cell types or may trigger different responses.

### Concluding remarks

The biology related to extracellular hsp is in exponential growth, leading to the discovery of more processes activated by these molecules. The multiple roles that have been associated with hsp, inside and outside cells, are remarkable. So far, we have gained a great deal of knowledge regarding the mechanisms for hsp export, which are likely to comprise multiple pathways. Similarly, it is evident that extracellular hsp come in different flavors, such as membrane-bound and membrane-free, each one with a specific systemic function. It could be envisioned that more mechanistic details for the release and function of hsp will be discovered within the next few years. It is amazing how far these disregarded initial discoveries have driven research on the stress response. However, we can expect more controversy to continue to arise, since it has been a common feature of this field.

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