

Exosomal Heat Shock Proteins as New Players in Tumour Cell-to-cell Communication

Review Article

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Received 19 Mar 2014; Accepted 23 May 2014

DOI: 10.5772/58721

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Abstract Exosomes have recently been proposed as novel elements in the study of intercellular communication in normal and pathological conditions. The biomolecular composition of exosomes reflects the specialized functions of the original cells. Heat shock proteins (Hsps) are a group of chaperone proteins with diverse biological roles. In recent years, many studies have focused on the extracellular roles played by Hsps that appear to be involved in cancer development and immune system stimulation. Hsps localized on the surface of exosomes, secreted by normal and tumour cells, could be key players in intercellular cross-talk, particularly during the course of different diseases, such as cancer. Exosomal Hsps offer significant opportunities for clinical applications, including their use as potential novel biomarkers for the diagnoses or prognoses of different diseases, or for therapeutic applications and drug delivery.

Keywords Extracellular Vesicles, Heat Shock Proteins, Cell Communication

1. Introduction

1.1 Cell-to-cell communication

Cellular communication is used by multicellular organisms to organize and coordinate the activities and the development of various organs and tissues [1]. In order to maintain cellular homeostasis or to respond to pathogens in the extracellular milieu, cells often exchange information through direct cell-to-cell contact or by secretion of soluble factors, either via ligand-receptor interactions or cellular 'bridges', such as nanotubes [2]. The cells interact with other cells through membrane surface molecules or by secreting several types of molecules such as soluble proteins, amino acids, fats,

steroids and gas. These molecules can activate the target cells by interacting with the cell surface receptors [1]. Recently, it has been shown that cells can also communicate through the direct exchange of nucleic acids. New evidence has shown that circulating miRNAs may be important in intercellular communication; in particular, they may induce gene silencing in the target cells [3, 4]. In addition to soluble molecules, cells can also send information through cell junctions and adhesion contacts, which can act within the same cell in which they are produced, in neighbouring cells, or even over long distances in an endocrine manner [5]. In the past two decades, another mechanism for intercellular communication has emerged involving the intercellular transfer of extracellular vesicles (EVs) [1]. Abundant evidence has validated a newly identified mechanism of intercellular interaction through lipid vesicles, in which phospholipid-enclosed vesicles are released into the extracellular environment that can bind the specific receptor to the target cells, and vesicles are internalized by recipient cells [6]. The release of EVs is a well-conserved evolutionarily mechanism that cells use to exchange bioactive proteins, lipids and nucleic acids. EVs released from cells are heterogeneous in origin and size, and include those derived from the endosomal membrane cell compartment, released by exocytosis after the fusion of multivesicular bodies with the plasma membrane, as well as those formed by direct budding of the plasma membrane [2, 7].

EVs were first characterized in hematopoietic cells. In 1967, Wolf observed these subcellular fractions ("microparticles"), which he described as "platelet dust" using electron microscopy [8]. Initially, it was thought that EVs were a mechanism of the depletion of the cytoplasm and a specific function of membrane reticulocytes. Indeed, EVs are rich in reticulocyte-specific proteins, transferrin receptor and devoid of some key plasma membrane proteins [9]. Increasing evidence supports the notion that each cytotype produces EVs (including T cells, B cells, dendritic cells, platelets, epithelial cells and cancer cells), which are essential players in intercellular communication and that they establish the ability of a cell to sense and adapt to environmental alterations [10, 13].

EVs are composed of a lipid bi-layer and contain multiple functional molecules derived from the cytosol of the donor cell, such as proteins (both transmembrane and luminal), lipids, RNAs, non-coding RNAs, microRNAs and retrotransposon elements [14]. EVs constitute a heterogeneous population that differs in cellular origin, size, morphology, antigenic composition and functional properties. They are classified into various categories based on their size and composition, for example, exosomes (40–100 nm), apoptotic bodies (>800 nm) [15],

microparticles (0.1–1 µm), prostasomes (50–500 nm) and tolerosomes (~40 nm), factors that create confusion in the nomenclature [16]. In addition, isolating them is extremely difficult. In fact, in recent years, researchers have tried to improve the various EV isolation protocols [1]. In many past studies, EVs were isolated by differential ultracentrifugation, depending on their size and density, but isolation protocols have not been definitively standardized. Following differential ultracentrifugation, a complementary characterization procedure using biochemical markers and electron microscopy imaging techniques is essential [13]. Among the large group of EVs, exosomes have been most studied to date, because of their involvement in both pathological and physiological events as mediators of cell-to cell communication [16].

2. Exosomes

As described by Pan in 1983, it was initially thought that exosomes could be a mechanism for shedding the cytoplasm in maturing sheep reticulocytes [17]. In fact, exosomes are cell-derived vesicles that are secreted by all cell types and are also present in many body fluids such as blood, urine, cerebrospinal fluid, breast milk, saliva, bronchoalveolar lavage fluid, ascitic fluid and amniotic fluid [18]. Exosomes are released into the extracellular space after the merging of late endosomes with the cell membrane. Early endosomes become part of multivesicular bodies (MVBs), which undergo a maturing process that provides a gradual change in protein composition of the vesicles (intraluminal vesicles (ILVs)). During this maturation process, the vesicles accumulated in the MVBs, can have three potential outcomes: 1) they may merge with the lysosomes, causing protein content degradation (e.g., in the case of receptors); 2) they may constitute a temporary storage compartment; 3) they may blend with the plasma membrane, releasing exosomes. Therefore, exosomes correspond to the intraluminal vesicles of MVBs. MVBs merge with the plasma membrane, resulting in exocytosis of the vesicles contained in MVBs; as such, vesicles maintain the same topological orientation as the plasma membrane [1, 19].

The endosomal sorting complexes required for transport machinery (ESCRT) are involved in exosome biogenesis and in their loading. Different evidence sources support the idea that ESCRT could assist in the sorting of ubiquitinated cargo proteins at the endosome membranes. The ESCRT-associated protein ALIX (apoptosis-linked gene 2-interacting protein X) can regulate this function [20]. Likewise, some evidence assumes that the sorting of proteolipid molecules to intraluminal vesicles functions independently of ESCRT. For example, in dendritic cells, during cognately antigen-specific CD4+ T cell interaction, the sorting of MHC II to

exosomes occurs independently from MHC II ubiquitination and MHCII can be incorporated into detergent-resistant protein complexes of intraluminal vesicles, which are secreted as exosomes and transferred to the interacting T-cells [21]. Another alternative cargo selection, independent from the ESCRT mechanism, occurs through lipid affinity, which requires sphingolipid ceramide and depends on raft-based microdomains [22-24].

Exosomes exhibit specific cell-type dependent content. It has been reported that their protein composition is similar to that of proteins found in plasma membranes, as well as in the endocytic or subcellular compartments of source cells and includes membrane proteins such as annexins [25]; cytoskeletal proteins (tubulin, actin) [26]; lysosomal markers (CD63, LAMP-1/2); enzymes [27]; death receptors (FasL, TRAIL) [28]; cytokines [29-31]; HLA class I/II [32] and some heat shock proteins (Hsps) [33, 34]. It has been demonstrated that exosomes derived from various cell types contain a wide variety of RNA, including mRNA, miRNA, rRNA and tRNA [35-41]. The RNA present in exosomes has been termed exosomal shuttle RNA (esRNA) [39] and can be transferred to recipient cells where they modulate cells' genetic expression [41]. Exosomes might constitute an exquisite mechanism for local and systemic intercellular transfer, not only of proteins, but also of genetic information in the form of RNA [39].

Depending on the source cell, many different functions have been attributed to exosomes. They are involved in cell-to-cell information transfer [42], immune response [43], inflammation [44], coagulation [45], stem cell activation [46] and programmed cell death [47]. Exosomes can participate in cellular responses against stress. Clayton and colleagues [48] showed that exposing B-cell lines to heat stress results in a marked increase of Hsps expression by exosomes and in an increase in the quantity of exosomes produced. Given that exosomes can mediate the transfer of specific molecules, they may play a role in intercellular transmission in disease pathogenesis, including tumour development, viral infections and neurological diseases. For example, exosomes might carry viral proteins from the infected cells from which they are released, thereby playing a part in the intercellular dissemination of viral vectors, or in clearing viral proteins from infected cells [49, 50]. Most studies have that many tumour cells release a large amount of EVs and those tumour-derived vesicles can carry proteins, lipids and nucleic acid that contribute to cancer progression [51]. In the case of many tumours, such as ovarian carcinoma, prostate cancer and pancreatic cancer, high levels of exosomes have been reported and these data suggest that exosomes could be important diagnostic and therapeutic tools [52-55]. Furthermore,

neuronal exosomes can mediate the transfer of misfolded proteins, causing a transmission mechanism of systemic amyloidoses in neurodegenerative diseases [56, 57]. All these data show that exosomes are important players in various physiological and pathological processes, and could be useful for both diagnostic and therapeutic applications. Some evidence obtained with exosomes released by human cancer cells support the existence of at least two entirely different mechanisms through which exosomes may interact with target cells. One is mediated by interaction of a ligand (often expressed on the exosome membrane) and its receptor (often expressed on the cell plasma membrane). This was clearly demonstrated for death-receptors/ligands interaction, which always led to the triggering of cell death [58-60]. However, exosomes may fuse with the plasma membrane of the target cells, in turn transferring their content to the cell cytoplasm and possibly fusing with internal vesicles, too [61]. The exosomes taken up by target cells may well have an effect, as it has been shown for NK cell-derived exosomes [60], suggesting that exosomes can be used as real effectors of the natural immune response against either tumours or foreign agents [62].

3. Hsps and exosomes

Molecular chaperones are a group of proteins conserved during evolution and are involved in the maintenance of other "client" proteins in folded and active conformations in all cellular organisms [63-65]. These chaperones protect the proteome from the dangers of misfolding and aggregation by facilitating protein folding, complex assembly and refolding of partially denatured proteins; additionally, they also drive protein translocation across membranes and in the case of protein damage, toward degradation [58, 59]. Chaperonology is the science that studies molecular chaperones and pathological conditions in which chaperones become pathological factors, known as chaperonopathies. Chaperone therapy involves the use of chaperones in the treatment of chaperonopathies [63, 64]. Most Hsps are molecular chaperones with crucial functions in the biosynthesis, folding/unfolding, transport and assembly of other proteins [63-66]. They are classified into families by their molecular weights: Hsp100, 90, 70, 60, 40 and the 'small Hsps', which includes Hsp27 [65]. Hsps were initially described as a group of proteins that are induced by heat shock, as well as by other stressors [67]. The expression of Hsps is induced in response to a wide variety of stress conditions, such as hypoxia, ischemia, heavy metal or ethanol exposure and infections [68]. Interest in these molecules has increased in recent years due to many studies having indicated that these proteins are involved in many physiological mechanisms in normal cells, such as DNA replication and gene expression regulation [63]. Several mechanisms are responsible for the cytoprotective

effect of Hsps [69]. Additionally, it has been demonstrated that Hsps have other roles, such as participation in immune system regulation [70, 71], cell differentiation [72], apoptosis and carcinogenesis [73-75]. The levels of many Hsps are elevated in various types of cancer and Hsp overexpression suggests a poor prognosis in terms of survival and response to therapy in some types of cancer [65]. Numerous studies have shown that Hsps are involved in cell transformation, metastasis formation and multidrug-resistance development [65, 74, 75]. Furthermore, several studies have reported that elevated levels of Hsps can protect malignant cells against therapy-induced apoptosis [65,69]. Hsps are traditionally considered intracellular molecules, but many studies have shown that they can also appear in extracellular locations or in the blood [76- 81]. Extracellular or membrane-bound Hsps can mediate immunological functions and may act as a potent danger signal, activating the immune system response [82]. Several years ago, some researchers hypothesized that exosomes may provide a secretory pathway, allowing cells to actively release specific Hsps [48, 83]. Exosomes are important in cell-to-cell communication; on the other hand, they are also considered to be key players in intercellular cross-talk [82]. Recent studies have validated this hypothesis by demonstrating that specific members of the Hsp family, such as Hsp70, Hsp90 and Hsp60, can

be secreted by cancerous cells via the exocytotic pathway (Table 1) [13, 33, 84, 85].

Hsp70 and Hsp90 are classic cytosolic chaperonins and normally fulfil a cytoprotective role inside cells [89]. Hsp70 is actively secreted by different types of cells through non-classical protein secretory routes, including exosome pathways [84]. Extracellular Hsp70 exert immunomodulatory effects and play a key role in the immune response to cancer cells [85]. For example, microvesicles containing Hsp70 on their surface activate macrophages [90], or natural killer cells [86, 87] and play an important role in the regulation of vascular homeostasis [84]. Hsp90 α is released by invasive cancer cells via exosomes and its release enhances cancer cell migration [88]. In recent years, new data has revealed new extracellular roles for Hsp60. Hsp60 is considered a mitochondrial protein that is, together with its co-chaperone Hsp10, essential for mitochondrial protein folding [74]. There is increasing evidence localizing Hsp60 outside of the cells, where it mediates interaction between immune cells and other body tissues [91]. In addition, much recent experimental evidence has demonstrated that Hsp60 can be localized in extramitochondrial sites [65]. In particular, it has been detected in the cytosol [92], intracellular vesicles [88], on the surface of normal and tumour cells [33, 93] and in

Tumoural Exosomal Hsps	Tumoural Cells Origin	Functions	Ref.
Hsp70	Human Erythroleukemia cells	Immunomodulatory functions	[85]
	Breast carcinoma cells		[85]
	Human pancreas carcinoma cells	Stimulation of migration and cytolytic activity of natural killer cells	[86]
	Human colon carcinoma cells		[87]
Hsp90	Human hepatocellular carcinoma cells	Stimulation of migration and cytolytic activity of natural killer cells	[87]
	Glioblastoma cells	Cancer cells motility	[88]
	Fibrosarcoma cells		[88]
	Breast cancer cells		[88]
Hsp60	Human Erythroleukemia cells	Immunomodulatory functions (?)	[13]
	Human lung adenocarcinoma		[13]
	Human mucoepidermoid bronchial carcinoma		[13, 33]
	Human hepatocellular carcinoma cells	Stimulation of migration and cytolytic activity of natural killer cells	[87]

Table 1. Origin and hypothetic functions of tumoural exosomal Hsps

blood [76]. In the cytosol, Hsps may play two distinct roles, given the numerous evidence implying that Hsps have pro-survival effects, while under different conditions they have been shown to have pro-apoptotic effects [92]. It is known that circulating Hsp can have immunosuppressing or immunostimulating effects, depending on the interaction between Hsps and cells or immune system components. For example, Hsp60 has been found in the blood of patients with Hashimoto's Thyroiditis (HT) and its presence may be involved in HT pathogenesis via an antibody mediated immune mechanism [94]. Moreover, extracellular Hsp60 can interact with a variety of receptors present on the surface of plasma cells, such as TLR, CD14, CD40 and CD91 [59]. Furthermore, Hsp60 appears to be involved in the activation of macrophages and neutrophils in patients with chronic lung diseases [95]. Levels of Hsp60 are increased in many types of tumours and it has been hypothesized that Hsp60 overexpression has an important role in cancer development and progression [65]. The use of Hsp60 as a biomarker for disease has recently been proposed and some researchers are studying the use of potential Hsp60 inhibitor agents in the treatment of certain diseases, including cancer [64, 96]. In heart failure, Hsp60 is released by cardiomyocytes and its presence in the serum may be correlated with the severity of the disease and cardiovascular risk [77, 97]. Moreover, Gupta and Knowlton [98] demonstrated that Hsp60 is released by adult cardiomyocytes through an exosome-mediated process in both the basal state and following mild stress. Another group of researchers demonstrated that fibrosarcoma cells release Hsp60 through the conventional endoplasmic reticulum Golgi protein transport pathway [99]. More recently, our research group showed that Hsp60 is released by tumour cells and not by normal cells, and that the mechanism of release is mediated by an unconventional secretion mechanism, i.e., the lipid raft exosome pathway [13]. These findings suggest a new role for extracellular Hsp60 in the cross-talk between tumour cells and the immune system [33, 99]. In fact, the expression of Hsp60 on the surface of exosomes released by tumour cells may be considered as a danger signal for the immune system [33]. Further studies are certainly needed to explain the unusual exosome membrane localization of Hsps [33, 100]. Experimental data show that exosomal Hsps may have opposing roles, that is, immunosuppressing or immunostimulating effects. These different effects depend on the interaction between exosomal Hsps and cells or immune system components. For example, it has been demonstrated that the histone deacetylase inhibitor, MS-275, can significantly alter the immune molecule content and categories in exosomes of hepatocarcinoma cells; in particular, treatment with MS-275 increased the expression of Hsp70. Exosome modification by MS-275 can significantly increase the cytotoxicity of NK cells and

the proliferation of PBMC, determining a reduction in tumour growth [101-104]. On the other hand, designing inhibitors of Hsp-associated exosomes may be useful to hindering the dissemination of metastases [88]. Moreover, the presence of Hsps associated with circulating exosomes can be evaluated and monitored quantitatively in the blood of patients with tumours associated with over-expression of one or more Hsps.

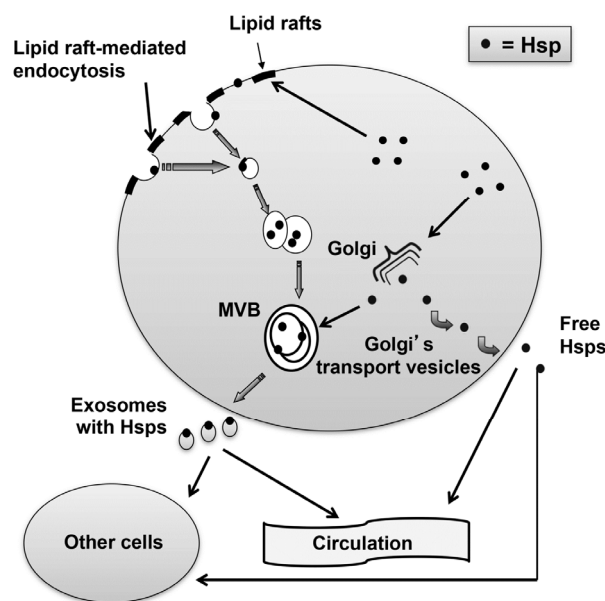


Figure 1. Pathways of secretion of heat shock proteins (Hsps) by tumour cells. Cytosolic Hsps can be released in free, soluble forms by Golgi transport vesicles or can be bound to exosomes. The latter is produced by multivesicular bodies (MVB) fusing with the plasma membrane of cells. Lipid rafts participate in Hsps release by exosomes, as they are internalized by endocytosis, which for various reasons that remain unknown reach the plasma membrane of tumour cells, and enter into MVB. Secreted Hsps may interact with other cells in the peritumoural environment or be released into the bloodstream.

4. Conclusions

Exosomes are currently considered to be bioactive vesicles that are useful in the study of normal biological functions, but also for understanding pathological conditions. The molecular composition of exosomes reflects the specialized functions of the original cells. Through exosomal ability to bind target cells and/or exchange molecules, they can modulate the activity of other cells. Hsps were originally described as intracellular molecular chaperones with a cytoprotective role. However, more novel functions have now been attributed to the Hsp proteins, depending on their localization. In particular, circulating Hsps (free or associated to exosomes) have immunological functions and may be involved in tumour progression [see Figure 1].

Hsps found on the surface of exosomes secreted by normal and tumour cells might be key players in intercellular cross-talk. There could be a novel and interesting link between Hsps and the immune system. Elsner et al. [101] demonstrated that Hsp70-positive exosomes released by tumour cells increase the NK cell activity against cell targets, resulting in reduced tumour growth. Therefore, exosomes can function as independent cell-to-cell carriers. Effectively, the immune response can be facilitated and enhanced by exosomes and their immunomodulatory molecules (such as Hsp70, 80, 90 and MHC class I molecules), released by the cell source into the blood [102]. Exosomes secreted by tumour cells and engineered to express specific Hsp molecules could improve antitumor immunity [105]. Therefore, engineered exosomes could be used as potential tumour vaccines or immunotherapeutic vesicles. Indeed, exosomes and their molecular cargo, including Hsps, are essential players in cell-to-cell communication and immunoregulation. There is significant potential for future clinical applications, including the use of Hsps as potential novel biomarkers for the diagnoses, prognoses and follow-up of different diseases, or for therapeutic applications and drug delivery. In particular, in light of new technical approaches, the levels of exosomes in human body fluids can be detected and quantified [106], and it is clear that exosomes have the potential to become important circulating biomarkers in the vast majority of human diseases [107]. Moreover, their intriguing capacity to shuttle molecules of various origins may well become one of the most important available drug delivery systems; they may therefore be of paramount importance for the future of nanomedicine [108].

5. Compliance with ethical research standards

Conflict of interest - The authors declare no conflicts of interest

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