Hsp70 in cancer: role of the membrane-anchored chaperone

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Abstract

Hsp70 is a highly conserved and inducible heat shock protein that belongs to the *HSP70* family of molecular chaperones and plays a central role in protein homeostasis. The main function of Hsp70 is to protect cells from physiological, pathological and environmental insults, as it assists an ATP-dependent manner the process of protein folding. Since Hsp70 provides critical cell survival functions, cancer cells are assumed to rely on this chaperone. Strong evidence suggests that Hsp70 is upregulated in different type of cancers and is involved in tumor growth, invasion, migration and resistance to anti-cancer therapy. Interestingly, this Hsp70 upregulation induces Hsp70 re-location into plasma membrane. In this review, the role of Hsp70 in cancer will be discussed focusing particularly on the extracellular membrane-bound Hsp70. The mechanism by which Hsp70 is translocated to plasma membrane of tumor cells and the recent discoveries of drugs targeting this Hsp70 in cancer therapy will be also highlighted.

Key words: membrane Hsp70, Cancer, Hsp70 translocation, Targeting Hsp70

1. Introduction

Heat shock protein-70 (Hsp70 also called Hsp72 or HSPA1) is a stress-inducible 70-kDa molecular chaperone of the highly conserved *HSP70* family [1]. The main physiological functions of Hsp70 within the cell are: (1) folding of both nascent and denatured proteins to native state, (2) refolding of intracellular aggregated proteins, and (3) degradation of aggregated proteins by ubiquitin proteasome system or via the process of molecular chaperones-mediated lysosomal autophagy [2]. It can also contribute to several cellular homeostatic events including the transportation of macromolecules like proteins and RNA, secretion, stimulation of signal transduction, regulation of transcription factors, cell division, migration, and differentiation (Fig. 1A) [3]. Structurally, Hsp70 has two distinct domains: a nucleotide-binding domain (NBD) and a substrate binding domain (SBD), where the unfolded peptide binds. Although the molecular mechanism by which Hsp70 assists the folding of denatured proteins into their native state still remains enigmatic, the common suggested mechanism is mainly assigned to the binding of the chaperone to hydrophobic residues of client proteins thereby preventing intermolecular interactions in an ATP-dependent manner [4,5]. In normal unstressed cells, Hsp70 is expressed at low or undetectable levels. However, its expression is upregulated in response to different types of cellular stress particularly protein damaging conditions like heat shock, oxidative stress, hypoxia, altered pH, and heavy metals [6,7]. A large number of studies including from us describe a cytoprotective role of Hsp70 toward different types of cell death like caspase-dependent/-independent apoptosis, necrosis or autophagic programmed cell death [8–15]. In addition, Hsp70 can be localized on the endolysosomal membrane of transformed cells and plays a major role in the resistance of the cancer cells to lysosomal cathepsine-induced cell death (Fig. 2B) [16]. In cells overexpressing Hsp70 such as cancer cells, Hsp70 can translocate to plasma membrane or can be extracellularly secreted. Although the function of

membrane Hsp70 is still largely unknown, it may provide an additional stress inducible role for Hsp70 such as stimulation of antitumor immune responses [10]. Extracellular Hsp70 has been shown to have a protective role against injury in different tissues such as liver and spleen since it stimulates the immune system to remove the unwanted cells from circulation [17]. As Hsp70 may confer survival advantage to tumor cells, several studies revealed Hsp70 major contribution to cancer cell resistant to chemotherapy. This includes *in vitro* resistance to cisplatin in prostate cancer [18], imatinib in chronic myeloid leukemia [19], topotecan and gemcitabine in fibrosarcoma [20], and oxaliplatin and 5-fluorouracil in colon cancer [21,22]. Therefore, Hsp70 inhibition can provide a novel therapeutic strategy to treat different types of cancers by targeting several oncoproteins and signaling pathways required for cancer initiation and progression. In this review, the role of membrane-anchored Hsp70, particularly in cancer, the mechanism by which Hsp70 translocated to plasma membrane and its pharmaceutical targeting in cancer therapy will be discussed.

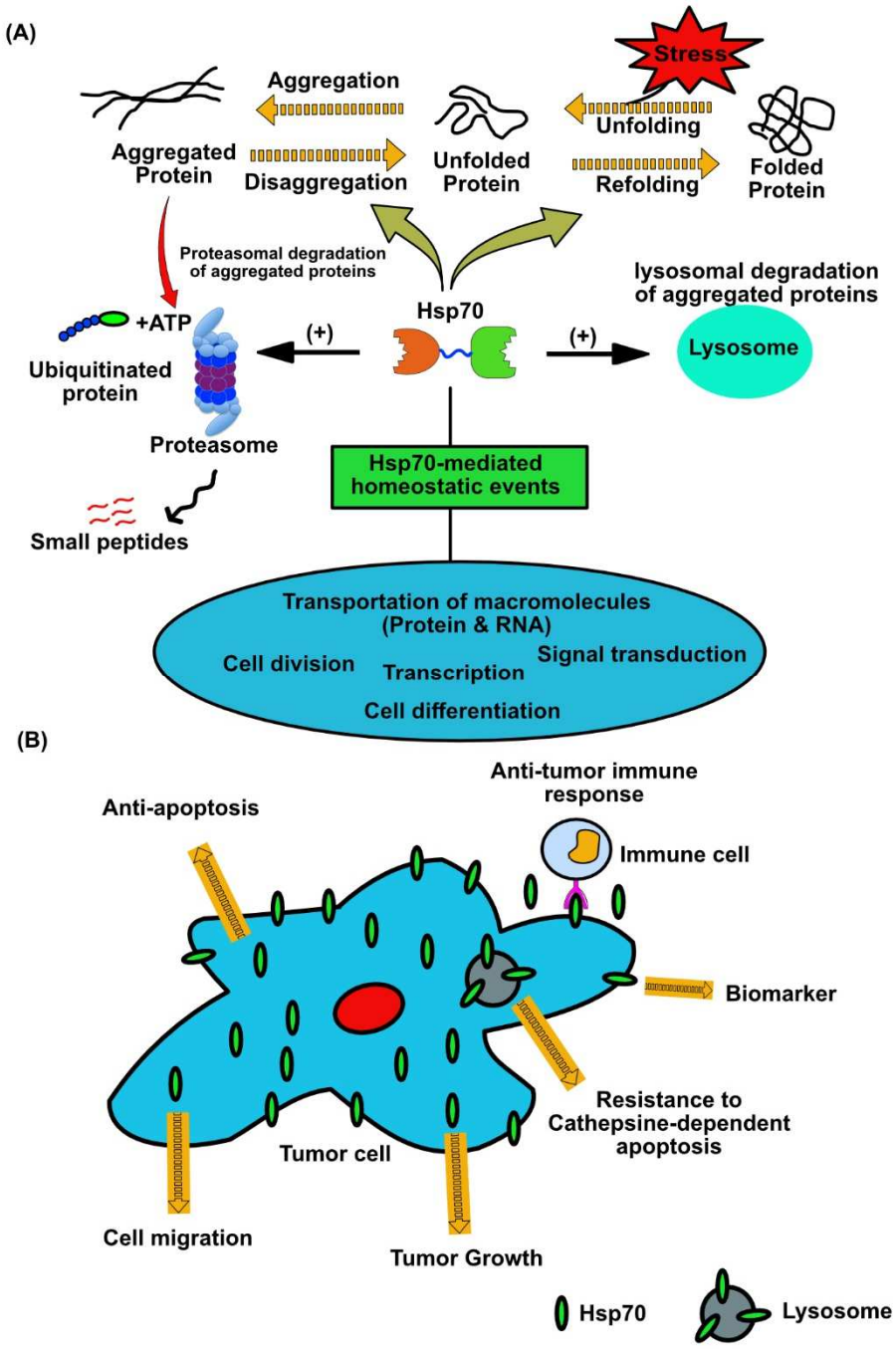

Fig. 1. (A) Illustrating the physiological functions of Hsp70: assists protein folding into its native form, proteasomal degradation of aggregated proteins, transport of client proteins across the cellular membrane, and some other homeostatic events. (B) The suggested role of Hsp70 in different type of cancers. Overexpression of Hsp70 in cancer cells can mediate several oncogenic events like anti-apoptotic response, antitumor immune response, tumor growth, and cell migration.

2. Overexpression of Hsp70 in tumor cells

Tumor cells because they have to re-wire their metabolism have a strong need of chaperones for their survival particularly Hsp70. Besides, tumor micro environment (TME) is considered to be a setting where cells are subjected to various types of environmental, physiological and pathophysiological stress including elevated level of oxidative stress, lack of nutrients, hypoxia and increased expression of mutant proteins. These factors are believed to play a central role in cancer initiation and progression [23] and contribute to the overexpression of Hsp70 in different types of cancer cells including hepatocellular carcinoma [24], cervical cancers [25], and acute myeloid leukemia [26].

Several *in vivo* and *in vitro* studies revealed that increased expression level of Hsp70 significantly stimulates both tumor growth [27–33] and cell migration [34–39] of different tumor cells (Fig. 2B). Correlation linking Hsp70 increased expression level to cancer development and progression prompted several research scientists to consider Hsp70 as a putative diagnostic biomarker for poor prognosis in cancer. For instance, Hsp70 was reported to be a potential diagnostic marker in hepatocellular carcinoma [40], prostate cancer [41], esophageal adenocarcinoma [42], lymph node metastasis in colorectal carcinoma [34], lung [43], ovarian [44] and breast cancers [45]. Hsp70 expression level has been associated to the clinical stage in melanoma [46], oral cancer [47], and to overall survival in bladder cancer [48]. Moreover, poor survival and prognosis in several tumors like acute myeloid leukemia, breast, and cervical cancers is greatly linked to high staining of Hsp70 in biopsies [49–51].

3. Membrane Hsp70

3.1 Mechanism of Hsp70 translocation to the cytoplasmic membrane

The expression of inducible Hsp70 in the membrane of various tumor cells, but not normal cells, was reported by several studies in response to the aforementioned stressful conditions [52–54]. The molecular mechanism by which Hsp70 is translocated to plasma membrane still remains under investigation. However, it is well reported that when anchored, the chaperone only leaves extracellular a 14-amino acid loop (aa 450–463) located in its C-terminal domain (TKDDNNLLGRFELSG, termed TKD) [55]. Hsp70 protein lacks a consensual sequence/signal peptide for the canonical (ER/Golgi) secretory pathway. Moreover, the translocation of Hsp70 across the plasma membrane of tumor cell is unaffected by the common inhibitor of the canonical secretory pathway such as brefeldin A (BFA) [56,57]. Here we summarize the suggested mechanisms for Hsp70 translocation to cancer cells plasma membrane (Fig. 2).

The influence of lipid rafts as an alternative molecular transport system on the delivery of Hsp70 to the cell membrane was studied. The isolated soluble and detergent-resistant microdomain (DRM) fractions from colon cancer Caco-2 cell line revealed the localization of Hsp70 in the DRM fraction. Exposure of cells to heat shock treatment obviously increased the Hsp70 expression and its efficient translocation to DRM. It has been also revealed that Caco-2 cells are able to release Hsp70 into culture medium under heat stress conditions. Treating with classical secretory pathway inhibitors did not affect this result. Interestingly, Hsp70 release was disrupted by treating cells with the cholesterol sequestering agent methyl-β-cyclodextrin (MβCD). This result supports the significant impact of DRM on Hsp70 membrane association and release [57]. Similarly, Hunter-Lavin and colleagues [58], also confirmed the Hsp70 release in PBMCs under normal culture conditions. Viable cell count and lactate dehydrogenase enzyme assay indicated that Hsp70 release occurs in the absence of cell damage. Indeed, Hsp70 release was inhibited by treating the cells with MβCD, but not the classical

secretory pathway inhibitor BFA. The interaction of Hsp70 with an artificial membrane bilayer mainly consisting of 1-palmitoyl-2-oleoylphosphatidylethanolamine (POPE) and 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) was investigated. The results displayed a rapid interaction of liposome-incorporated recombinant Hsp70 with the artificial membrane, accompanied by an opening of ion channel conductance. In addition, Hsp70 was detected on the surface of the plasma membrane of heat-stressed HepG2 using immunostaining with FITC conjugated cmHsp70 monoclonal antibody that targets the TKD peptide of Hsp70. These results indicate that Hsp70 might translocate to the plasma membrane within DRM [59,60]. It has been reported that both Hsp70 proteins, i.e. the stress inducible (Hsp70) and the constitutively expressed (Hsc70), have the ability to interact and to aggregate in an artificial liposome, mainly consisting of phosphatidylserine (PS) in a time and a protein concentration-dependent manners. These results suggested a role for the heat shock proteins in the proper folding of membrane proteins and in client proteins translocation across the membrane [61]. Liposome insertion assay detected the interaction of Hsp70 with liposomes enriched in PS. The authors also reported high molecular weight Hsp70 oligomers following insertion into the lipid bilayer. Moreover, Hsp70 accumulation on the liposomal surface was increased in the presence of PS-enriched saturated fatty acids. The results of this study suggested that Hsp70 interaction with the plasma membrane may favor a rigid membrane structure [62]. Hsp70 can specifically interact with PS moieties on the membrane of pheochromocytoma PC12 cells in the early apoptotic phase. This was accompanied by rapid Hsp70 integration into the cell membrane. Addition of Hsp70 to the medium resulted in a significant reduction of PC12 cell viability. Hsp70 binding to PS was disrupted following cell treatment with annexinV. This suggested a role for Hsp70 in the phospholipid flip flop during

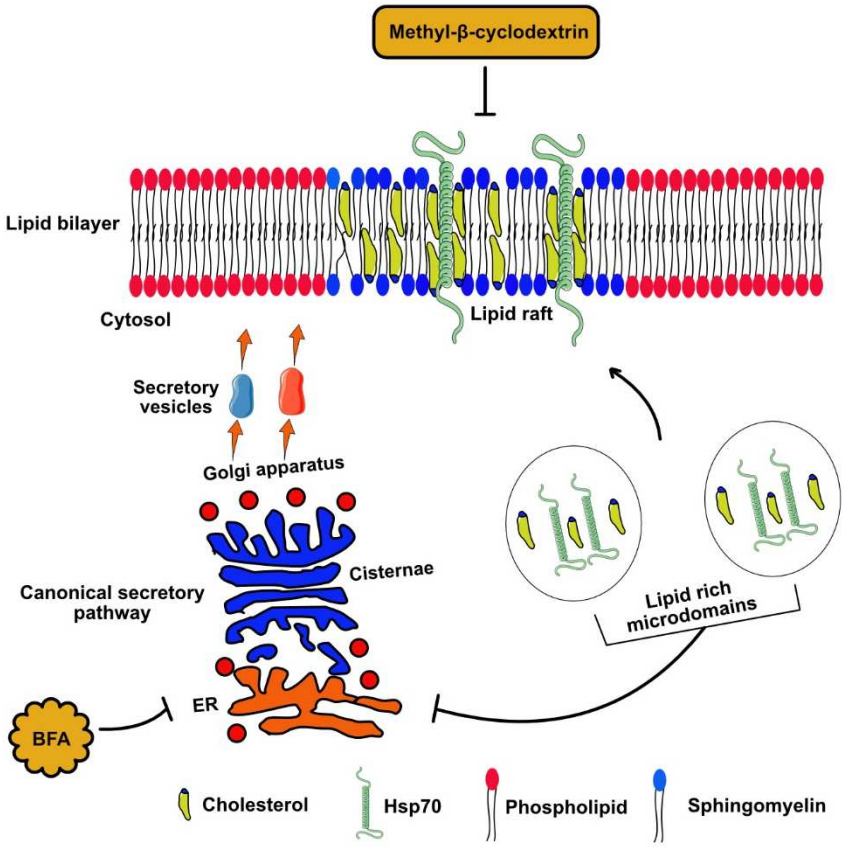

Fig. 2. Illustrating the suggested mechanism by which Hsp70 is translocated to plasma membrane. As Hsp70 lacks a consensual sequence necessary for its transportation across plasma membrane through the canonical secretory pathway, it is believed that Hsp70 can be transported within detergent-resistant lipid rich microdomains (DRM). Methyl-β-cyclodextrin can inhibit Hsp70 release, but not classical secretory pathway inhibitor like brefeldin A (BFA).

early apoptotic cell death [55]. It has been reported that the interaction of Hsp70 with liposomes depends on their lipid compositions. A weak binding of Hsp70 to the liposomal surface that mainly consisting of phosphatidylcholine (PC), was reported. Fluorescence microscopic analysis revealed the insertion of both tryptophan residues W90 and W580 of Hsp70 in the liposome bilayer hydrocarbon region. In contrast, Hsp70 was found to peripherally bind to a liposomal surface mainly consisting of phosphatidylserine, cardiolipin, or bis-monoacylglycero phosphate [63]. In heat-stressed primary and differentiated adipocytes, Hsp70 was found to be localized within a lipid droplet enriched in a triacylglycerol, cholesterol ester, and surrounded by a monolayer of phospholipids. Alkaline treatment of adipocyte revealed that Hsp70 may bind to

droplet surface monolayer through noncovalent interactions. Results suggested the essential role of Hsp70 stabilizing the droplet monolayer, protein transport to lipid droplet, and proper folding of denatured protein on the surface of lipid monolayer [64].

3.2. Membrane-anchored Hsp70 in tumor cells

Membrane Hsp70 in cancer cells was reported to have additional stress-inducible roles such as induction of cell survival and contribution to antitumor immune response. These properties are often associated to the membrane Hsp70 extracellular 14 amino acids TKD sequence [55,65,66]. In this context, the role of membrane-associated Hsp70 in the pathogenesis of cancer was investigated. Flow cytometry analysis revealed a significant increase of Hsp70 surface expression level on both dysplasia and carcinoma cells via *in vitro* enhancement of the cytolytic activity of NK cells in oral [67], colon cancer [68] and fibrosarcoma MethA tumors [69]. Increased expression level of another Hsp, Hsp90, was also observed in the membrane of poorly differentiated tumor cells as well as in advanced clinical stage of tumors (T3/T4). *In vitro* activation of NK cells by both Hsp70 peptide (TKD) and interleukin-2 (IL-2) followed by reinfusion of activated cells, greatly enhanced NK cells cytolytic activity against Hsp70 membrane-positive colon carcinoma cells [67]. Activation and maturation of dendritic cells were also found to be mediated by Hsp70 via the TRL4 pathway [70,71]. Elevated Hsp70 level enhances the proliferative responsiveness of T-lymphocytes toward Hsp70 positive in osteosarcoma cell lines [72]. It has also been reported that Hsp70 is localized on the endolysosomal membrane of CX2 human colon carcinoma cells and plays a major role in the resistance of cancer cells to lysosomal cathepsine-induced cell death. Depletion of Hsp70 significantly enhanced lysosomal membrane permeability and subsequent release of

hydrolyzing enzymes to the cytosol, suggesting a pro-survival function of Hsp70 in colon cancer [16].

3.3. Membrane Hsp70 in tumor-derived exosomes

Accumulating evidence suggests that Hsp70 can be secreted through extracellular vesicles (EVs), particularly exosomes. These are nanovesicles (50-200nm) generated within the endosomal compartment by most eukaryotic cells [73,74]. It is well known that tumor cells produce larger number of exosomes when compared to normal cells and the content of the tumor-derived exosomes (TDEs) is quite different from normal cells-derived exosomes. One of the specific surface biomarkers associated with TDEs is the stress protein Hsp70 [75].

It has been reported that Hsp70 interacts with death silencing domain (Bag4) on the surface of colon (CX+) cancer cells-derived exosomes. Localization of Hsp70/Bag4 on the exosomal surface significantly stimulated NK cells migration and cytolytic activity against a Hsp70-positive tumor [76]. Considering possible vaccination strategies against hepatocellular carcinoma, it has been reported that exosomes derived from the chemotherapeutic resistant cell line HepG2 possessed high amounts of Hsp70 both within exosomes and on exosomal membrane. Moreover, exosomes bearing Hsp70 showed immunoregulatory properties by stimulating NK antitumor response and production of granzyme B. Since expression of the inhibitory receptor CD94 was upregulated, the expression level of activating receptors CD69, NKG2D, and NKp44 was decreased [77]. The ability of TDEs to stimulate antitumor immune response was analyzed in an engineered myeloma (J558HSP) cell line. Cells were manipulated to express the endogenous P1A tumor antigen and membrane-bound Hsp70. It was revealed that exosomal Hsp70 derived from such tumor cell line stimulated the maturation of dendritic cells accompanied by an upregulation of CD40 and CD80 (Fig. 3). Furthermore, immunization of BALB/c inbred mice with TDEs enriched with

Hsp70 leads to stimulation of T-helper cells and CD8+ cytotoxic T-lymphocytes suggesting exosomal Hsp70 antitumor activity [78].

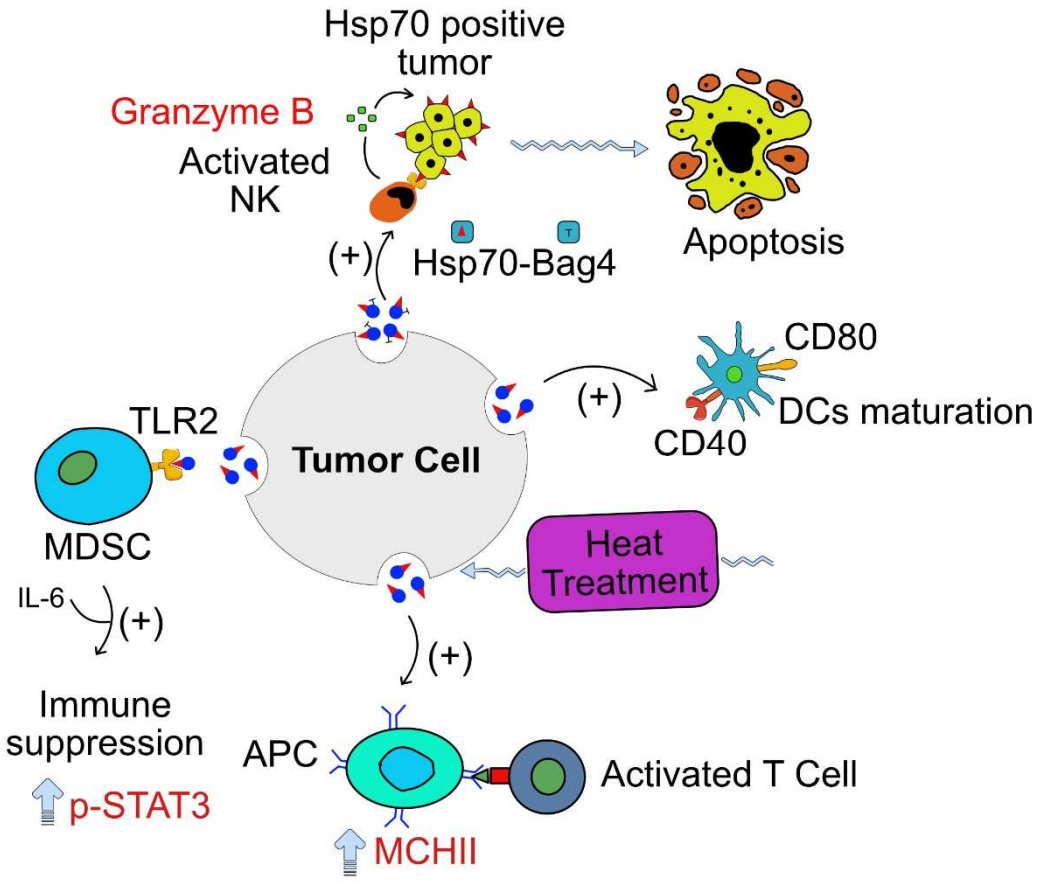

Fig. 3. Illustrating the suggested role of tumor-derived exosomal Hsp70 in cancer. The presence of Hsp70 on the membrane of TEDs may exert an anti-tumor immune response via NK cells activation, maturation of DCs-mediated T-cell cytotoxicity, and overexpression of MCHII on the surface of APCs. It can also enhance tumor growth via stimulation of the immune suppressive activity of MDSC and overexpression of phosphorylated STAT3.

Hsp70-enriched exosomes derived from heat-treated CT26 mouse colon carcinoma cells significantly enhanced antitumor effect of TDEs via the expression of MHC II in an *in vitro* antigen presenting cell model. Investigating the antitumor effect in an allogenic mouse model, authors found a stimulation of Th1-polarization followed by an increased production of IgG2a and Interferon gamma (IFN-γ) [79]. A study was conducted to test the immunomodulatory effect of tumor-derived exosomal Hsp70 on the suppressive activity of myeloid-derived suppressor cells (MDSCs). It was shown that TDEs enriched in membrane-bound Hsp70 triggered MDSCs activation. This occurred via association of Hsp70 to the

toll like receptor 2 (TLR2) on MDSCs, resulting in upregulation of phosphorylated signal transducer and activator of transcription 3 (STAT3) and autocrine secretion of IL-6 (Fig, 3). These effects were no longer observed if exosomes previously treated with a Hsp70 specific antibody or peptide aptamer binding to the extracellular domain (TKD) of membrane-bound Hsp70. Moreover, treatment of tumor cells with amiloride decreased exosomes production led to the enhancement of the antitumor effect of the chemotherapeutic drug cyclophosphamide [75]. TDEs from the supernatants of Renca cell cultures were found to exhibit more Hsp70 on the membrane. In accord with the previous reports, addition of these Hsp70-exosomes to MDSCs stimulated proinflammatory cytokines activation, tumor growth factors production, and tumor progression. However, this observation was totally reversed when exosomes were preincubated with an anti-Hsp70 antibody. The effect of exosomal Hsp70 on the stimulation of MDSCs suppressive activity via STAT3 phosphorylation was confirmed in renal carcinoma. This mechanism might provide a future therapeutic approach [80]. Our previous results indicated that several cancer cell lines including breast, lung, and ovarian cancer were able to produce high amount of exosomes when compared to their normal counterparts. Moreover, interferometric analysis of exosomes using the Hsp70 aptamer A8 indicated a significant Hsp70 expression on the surface of TDEs from a wide panoply of cancer cell lines, compared to their normal counterparts where membrane Hsp70 remained undetected. Combined treatment of tumor-bearing mice with cisplatin/A8 or 5-fluorouracil/A8, but not single treatments, induced a complete regression of tumor that was linked to the inhibition of Hsp70 immuno-suppressive activity, suggesting that Hsp70 role in MDSCs activation may contribute to its overall effect on tumor growth [22].

4. Membrane Hsp70 as a promising target in cancer therapy

The higher metabolic activity of tumor cells associated to the overexpression of oncogenes, mutated proteins and down regulation of tumor suppressor genes explains the essential role of chaperones like Hsp70 in tumor cells survival [81]. Targeting membrane Hsp70 can provide a novel strategy in cancer immunotherapy. Since it is well reported that the amount of Hsp70 in the membrane is dependent on the total cellular amount of Hsp70 (approximately 10% of cytosolic Hsp70 is anchored to plasma membrane [10,22]), here, we will discuss both direct inhibitors, which target the extracellular Hsp70 TKD domain, and indirect inhibitors that target intracellular Hsp70.

The first molecule described targeting specifically inducible Hsp70 was a neutralizing peptide, which contained the apoptosis inducing factor (AIF) domain that interacts with Hsp70. This peptide, known as ADD70 for AIF-derived decoy for Hsp70, was found to sensitize rat colon cancer cells (PROb) and mouse melanoma cells (B16F10) toward apoptosis-induced by cisplatin (see Fig. 4). ADD70 had the ability to bind the substrate binding domain (SBD) of Hsp70 blocking Hsp70-AIF association and subsequent cytoprotective activity [84,85]. 2-Phenylethynesulfonamide (PES), also known as Pifithrin-μ, is a chemical molecule that blocks Hsp70 by binding to its SBD thereby preventing Hsp70 association with several client proteins like APAF-1 and p53 and its co-chaperone Hsp40 [16]. The molecular mechanism by which PES triggers tumor cell death was mainly attributed to induction of apoptosis, degradation of the misfolded proteins and destabilization of lysosomal membrane [86]. The combined treatment between Pifithrin-μ and oxaliplatin greatly enhanced the cytotoxic effect of oxaliplatin against both colon (HCT116 and LoVo) and prostate (LNCaP, PC-3, and DU145) cancer cell lines [87]. In additions, PES displayed an obvious cytotoxic effect in several leukemia cell lines in combination with the histone deacetylase inhibitor vorinostat (SAHA) or with the Hsp90 inhibitor, geldanamycin analog (17-AAG) [88]. A similar interesting combinational effect

was obtained when PES was associated to the proteasome inhibitor bortezomib [89] or MG-132 to treat primary multiple myeloma cells [90]. MAL3-101 is an allosteric Hsp70 inhibitor targeting the nucleotide binding domain (NBD) by inhibiting its ATPase activity. It showed an anti-proliferative effect against breast (SK-BR-3) cancer [91], multiple myeloma [89], and Merkel cell carcinoma [92] cell lines. The adenosine-derived compound VER-155008, a specific Hsp70

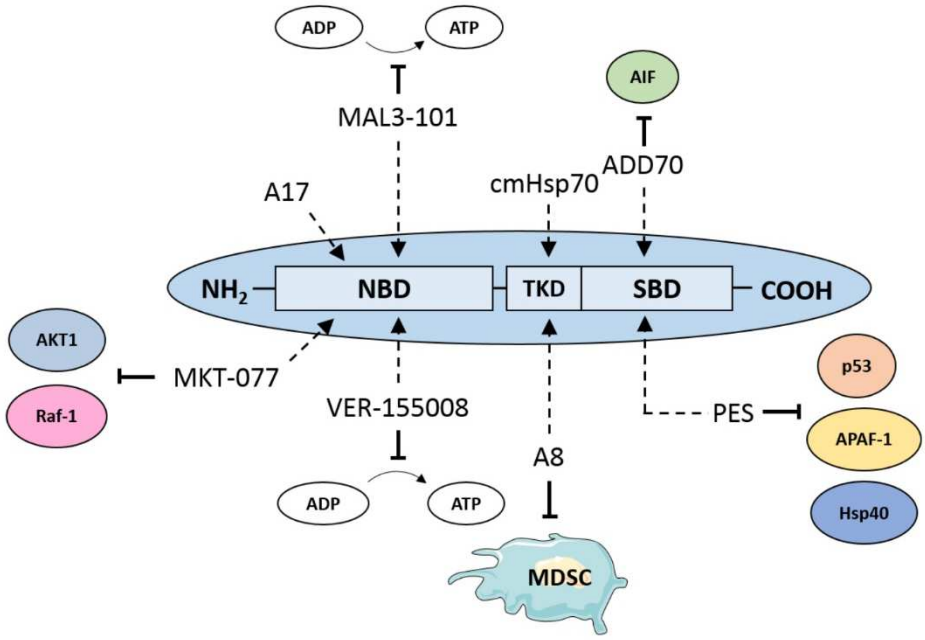

Fig. 4. Compounds targeting Hsp70 in cancer therapy. Most of the inhibitors bind to the nucleotide binding domain (NBD) blocking ATPase activity like MAL3-101, A17 aptamer, MKT-088, and VER-155008. The extracellular motif of membrane Hsp70 (TKD) is recognized by the monoclonal antibody cmhsp70 and the aptamer A8. Finally, Pifithrin (PES) and ADD70 target the substrate binding domain (SBD) of Hsp70.

inhibitor that blocks NBD, was found to induce apoptotic cell death in colon carcinoma cells [93], and to exert a significant reduction of cell viability in both melanoma [94] and colon cancer [93] cell lines, when combined with the Hsp90 inhibitor tanespimycin. The rhodacyanine dye analog of MKT-077 is also an allosteric Hsp70 inhibitor that binds the NBD. This compound revealed a marked cytotoxic effect in breast cancer MDA-MB-231 and MCF-7 cell lines via destabilization of the chaperone client proteins Akt1 and Raf1 and subsequent apoptosis induction [95].

In a more targeted approach, the monoclonal antibody cmHsp70 was designed to specifically bind the extracellular TKD domain of membrane Hsp70. This antibody showed an antitumor effect in colon tumor bearing BALB/c mice resulting in a significant reduction of tumor growth and an enhancement of overall survival. Moreover, these effects were associated to an increase of NK cells, macrophages, and granulocytes tumor infiltration. The cmHsp70 has also shown efficacy in combination with radiotherapy in NSCLC patients [10]. Peptide aptamers were also developed to inhibit Hsp70 as novel lead compounds in cancer therapy. For example, A8 is a peptide aptamer that binds to the extracellular TKD domain of membrane Hsp70. As a result, A8 blocks MDSCs activation by TDEs expressing Hsp70 on the surface. Treatment of tumor-bearing mice with A8 has been shown to induce a marked decrease of tumor growth by decreasing number and the activation of splenic MDSCs. Furthermore, this treatment has also been reported to improve the development of an antitumor immune response via enhancement of the number of anti-tumor macrophages (M1-like) and CD8+ T cells. When combining with chemotherapeutic treatments such as cisplatin or 5-fluorouracil, the antitumor effect of A8 was further improved [22]. A17 is another peptide aptamer that binds Hsp70 ATP-binding domain, has been shown to exert cytotoxic effects when combined to chemotherapeutic drugs cisplatin in different cancer cell lines and to induce cell death and tumor regression in B16F10 tumor bearing mice [96]. Studies combining A8, to develop an efficient anticancer immune response, and A17 to induce cancer dell death, are currently under investigation. Another phase I clinical trial investigated the stimulatory effect of Hsp70 peptide TKD along with interleukin-2 (IL-2) on NK cells in patients with advanced colorectal and non-small cell lung cancer (NSCLC). Results evidenced that NK cells killed Hsp70-positive tumor cells after activation with TKD and low doses of IL-2 [67].

5. Concluding remarks

The stress inducible molecular chaperone Hsp70 shows a high level of expression in a great variety of cancers, explaining why TDEs express Hsp70 in their membrane. The process by which a part of cytosolic Hsp70 re-locates to the plasma membrane is still a matter of debated issue, as the protein is deprived of a signal peptide enabling transport via the canonical secretory pathway. Several studies suggested Hsp70 transportation within lipid rich microdomains. Hsp70 can therefore be used as a diagnostic marker for cancers. Clinical results so far indicate that Hsp70 is present in tumor liquid biopsies, notably in circulating TDEs, and is associated with a poor prognosis. From a functional point of view Hsp70 has been involved in cancer initiation and progression. The signaling pathway induced by Hsp70 seems to depend on the type of cancer cells and stress conditions. This include uncontrolled cell proliferation, cytoprotective effect, enhanced cell survival and cell migration. The expression of Hsp70 on the surface of cancer cells was also reported to have additional roles in cancer development. Pharmaceutical targeting of Hsp70 has become an interesting trend towards the development of a novel therapeutic strategies against different types of cancer. Several compounds are now under preclinical evaluation. Molecules specifically targeting membrane Hsp70 (antibodies and peptide aptamers) might have particular interest. Those molecules block Hsp70 activation of MDSCs thereby inducing an efficient anti-cancer immune response. They could be used in combination with any anti-cancer therapy inducing Hsp70 (most chemotherapeutic drugs or Hsp90 inhibitors). Those peptides or antibodies could be used both for therapeutic and diagnosis purposes (to detect circulating Hsp70, particularly nanovesicules expressing Hsp70 on the membrane). These molecules may open new avenues for the use of Hsp70 as a target in cancer theranostics.

Author contributions

ME, JG, and CG conceived the idea for the review. ME and MC searched the literature and wrote the manuscript. ME and MC generated the figure panels. GC and VV edited the final version of the manuscript.

Conflict of interest statement

The authors declare no financial conflict.

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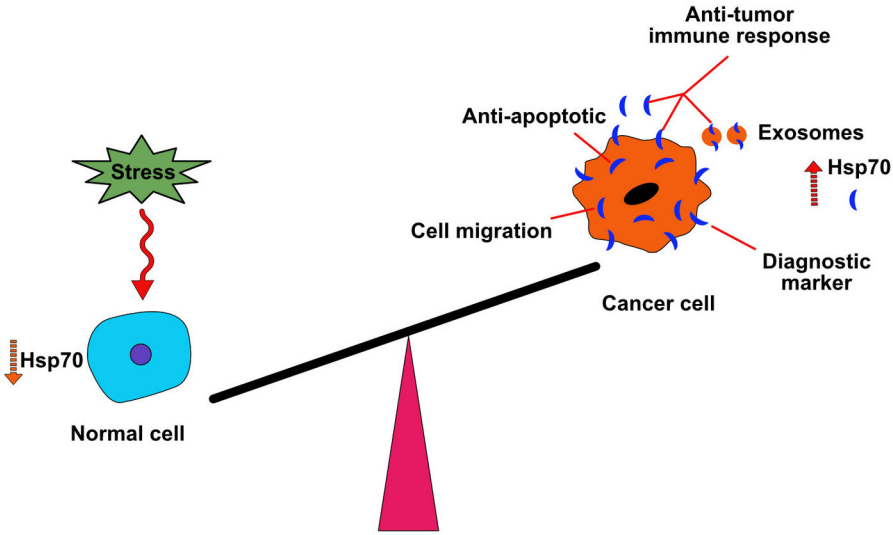
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