Targeting Hsp70: A possible therapy for cancer

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Abstract

In all organisms, heat-shock proteins (HSPs) provide an ancient defense system. These proteins act as molecular chaperones by assisting proper folding and refolding of misfolded proteins and aid in the elimination of old and damaged cells. HSPs include Hsp100, Hsp90, Hsp70, Hsp40, and small HSPs. Through its substrate-binding domains, Hsp70 interacts with wide spectrum of molecules, ranging from unfolded to natively folded and aggregated proteins, and provides cytoprotective role against various cellular stresses. Under pathophysiological conditions, the high expression of Hsp70 allows cells to survive with lethal injuries. Increased Hsp70, by interacting at several points on apoptotic signaling pathways, leads to inhibition of apoptosis. Elevated expression of Hsp70 in cancer cells may be responsible for tumorigenesis and for tumor progression by providing resistance to chemotherapy. In contrast, inhibition or knockdown of Hsp70 reduces the size of tumors and can cause their complete regression. Moreover, extracellular Hsp70 acts as an immunogen that participates in cross presentation of MHC-I molecules. The goals of this review are to examine the roles of Hsp70 in cancer and to present strategies targeting Hsp70 in the development of cancer therapeutics.

Key words: Hsp70, apoptosis, cancer therapeutics, immunogenicity

Introduction

In all organisms, the heat-shock response, induced by a wide range of stimuli, increases expression of a family of proteins called heat-shock proteins (HSPs) [1, 2] that act as molecular chaperones and, for cells under stress, exhibit cytoprotective properties [3, 4]. Stress beyond a certain threshold induces misfolding and aggregation of proteins and disruption of regulatory complexes [5]. In mammalian cells, the chaperone functions of HSPs maintain and restore cellular homeostasis. Principal heat-shock proteins that have chaperone activity belong to five conserved classes: Hsp33, Hsp60, Hsp70, Hsp90, Hsp100, and the class of small heat-shock proteins (sHSPs). Members of the HSP family, either expressed constitutively or regulated inductively, are transported to various cellular compartments. HSPs of large molecular weight are ATP-dependent molecular chaperones; small HSPs act in an ATP-independent manner. Under physiological conditions, the chaperones also assist in signaling and protein trafficking. However, harmful assaults on cells increase the demand for HSPs (Hsp70 in particular). Among the HSPs, Hsp70 has strong cytoprotective properties. Stress-induced expression of Hsp70 protects cells from lethal injuries and therefore provides a barometer of how pro-apoptotic stimuli elicit a protective response [6-9].

Hsp70 is also involved in cell growth, cell proliferation, and erythroid differentiation during formation of erythrocytes. Accumulation of this protein in erythroblast nuclei protects GATA-1, a transcription factor necessary for erythropoiesis [10]. Malignant cells express higher levels of Hsp70 than normal cells, and high Hsp70 expression is indicative of a tumorigenic phenotype that typically resists chemotherapy and programmed cell death [11-13]. Moreover, Hsp70 interferes at various points on the apoptotic pathways, preventing stress from inappropriately inducing cell death. In addition to its anti-apoptotic function, Hsp70 has an immunomodulatory effect and shows cross presentation with the MHC-I molecule [14]. Hsp70 activates both arms of the immune system (innate and adaptive) and acts as a potent immunomodulator. The goals of the review are to focus on the tumorigenic effects of Hsp70 and potential strategies for treatment of cancers and to assess the immunological role of Hsp70 in cross presentation.

Hsp70: cellular lifeguard

During periods of stress, human cells produce high levels of Hsp70, constitutively expressed as Hsc70, mitochondrial Hsp75, and GRP78, which are found in the endoplasmic reticulum. Hsp70

functions as an ATP-dependent molecular chaperone that assists proper folding/refolding of newly synthesized polypeptide chains, assembly of complex structures, and the transport of proteins through the cell membrane [15-20]. Structurally, Hsp70 possesses a peptide binding domain (PBD) and an N-terminal ATPase domain (ABD) (Fig. 1). The EEVD motif in the carboxyl terminal of the PBD is responsible for substrate binding/refolding (Fig. 1). The ABD induces release of client proteins and accomplishes ATP hydrolysis. Upon ATP binding, a proline residue in the ATPase domain induces a conformational change and causes hydrolysis [21]. Several associated co-chaperones also bind to Hsp70 and regulate its chaperone function. The Hsp70-associated co-chaperones are of three types: J-domain co-chaperones (Hsp40 binds to ABD in Hsp70 and stimulates low ATPase activity for this chaperone); the nucleotide exchange factors Bag-1, Hsp110, or HspBP1 catalyze the release of ADP and complete the Hsp70 ATPase cycle; the TPR domain (Hop, CHIP) chaperones bind to the C-terminal EEVD motif of both Hsp70 and Hsp90 [22-24]. These are essential for the assembly of Hsp70 and Hsp90 complexes (Fig. 1) [24].

Please incorporate Fig. 1 here

Stress-induced expression of Hsp70 allows cells to cope with large amounts of unfolded and/or denatured proteins. Further, the Hsp70 housekeeping functions involve transportation of precursor proteins into cellular compartments; protein folding (cytosol, endoplasmic reticulum, and mitochondria); degradation of unstable proteins and protein complexes; control of regulatory proteins; and protein refolding (Fig. 2) [25].

Please insert Fig. 2 here

Hsp70: an anti-apoptotic protein

Apoptosis is essential for embryogenesis, development, and maintenance of cellular homeostasis [26, 27]. Anti-cancer drugs induce it through a variety of mechanisms [28]. Either an intrinsic (mitochondria-dependent) or extrinsic (death receptor) pathway facilitates the process. Caspase-3, the effector caspase, is involved in both pathways and is responsible for apoptosis in target cells [29].

In response to a death signal, activation of the intrinsic apoptotic pathway results in altered expression of the Bcl2 family of proteins [30]. These include anti-apoptotic (Bcl2 and Bcl-xL); pro-apoptotic (Bax, Bcl-Xs, **and** Bak); and BH₃-only proteins, which function upstream to Bax, Bak, and Bcl-Xs [30-32]. Downstream to mitochondria, released cytochrome c interacts with cytosolic apoptotic protease activation factor-1 (Apaf-1) to form the apoptosome with caspase-9, which leads to activation of caspases [33, 34]. In addition to Bcl2 proteins, Smac/Diablo and Htra2/Omi induce apoptosis by blocking inhibitory apoptotic proteins (Fig. 3) [35-37].

Please insert Fig. 3 here

Extrinsic apoptosis (the death receptor pathway) is triggered by plasma membrane-associated proteins of the TNF-family of receptors, which lead to activation of caspase-8/10 in the death-inducing signaling complex (DISC) [38]. Caspase-8 either directly activates executioner caspases (caspase-3/6/7) or cleaves Bid into t-Bid, which connects the extrinsic and intrinsic apoptotic pathways [39-41]. In apoptotic signaling, highly expressed Hsp70 interferes at several points, including the release of cytochrome c, activation of caspases, accumulation of misfolded proteins, generation of reactive oxygen species, and DNA fragmentation [13, 42]. Further, inhibition/knockdown of Hsp70 increases sensitivity of cells to apoptosis [43-45]. Hsp70 inhibits caspase activity directly or indirectly, thereby blocking the intrinsic and extrinsic apoptotic pathways through interaction with key apoptotic proteins at three levels: up-stream to mitochondria, at mitochondria, and post-mitochondria (Fig. 3). Thus, Hsp70 directly or indirectly modulates the intrinsic and extrinsic apoptotic pathways.

Hsp70 and the intrinsic apoptotic pathway

Expression of Hsp70, an evolutionary-conserved protein involved in apoptotic signaling, increases the survivability of cells under stress. Cells with Hsp70 knockdown are sensitive to apoptosis [46]; over-expression of Hsp70 inhibits apoptosis, acting either downstream or upstream to mitochondria.

Furthermore, Hsp70 interacts with nerve growth factor and platelet-derived growth factor and enhances the survival of cells through activation of the PI3K signaling pathway. Activated PI3K leads to activation of ser/thr kinases (Akt/PKB) to generate a growth factor-mediated survival

signal. Akt kinase targets Bad and caspase-9 in the apoptotic cascade [47-50]. In K562 cells, Hsp70 stabilizes the Akt/PKB complex [51]. In zebra fish, HspA12B, a member of the Hsp70 family, is required for vasculature development, and, by sustaining Akt activity, is involved in endothelial cell migration and tube formation [52]. Thus, members of the Hsp70 family are implicated in the regulation of cell survival and differentiation. Hsp70 is also involved in the re-phosphorylation and stabilization of proteins through the priming of non-phosphorylated protein kinases [53]. In NIH3T3 cells, Hsp70 inhibits a stress-activated kinase (apoptosis signal regulating kinase-1), and its down-regulation facilitates production of H₂O₂, ASK-1 activation, and apoptosis [54]. In addition, Hsp70 binds to C-Jun N-terminal kinase (JNK) and blocks its ATP-dependent activation[55]. Mouse embryo fibroblast (MEF) Hsp70.1^{-/-} cells are resistant to JNK-mediated apoptosis, and AEG 3482, an anti-apoptotic compound, inhibits JNK activity by inducing expression of Hsp70 [56]. In primary cultures of IMR90 human fibroblasts, Hsp70 inhibits P38 kinase [57], which participates in a signaling cascade controlling cellular responses to cytokines and stress.

Hsp70 affects the expression of transcription factors associated with proteins of the Bcl2 family [58]. Bcl2 and Bax are the targets of the tumor suppressor protein, p53. In response to DNA damage, transcription of Bcl2 is repressed, and Bax is induced [59]. Tumor cells often have a mutated p53, which forms a stable complex with Hsp70/hsc70 [60]. Stress-mediated expression of Hsp70 inhibits the nuclear import of p53 [60, 61]. However, how Hsp70 regulates NF- κ B-function is still poorly understood. Cytosolic Hsp70 could inhibit NF- κ B expression, and membrane-bound Hsp70 could induce this transcription factor [62]. In response to similar stimuli, cytosolic and membrane-bound Hsp70 are stimulated [63]. In endothelial cells, stress-induced expression of Hsp70 facilitates TNF- α -mediated apoptosis by blocking the NF- κ B survival pathway [64]. Furthermore, Hsp70 blocks NF- κ B-activation through inhibition of I- κ B- α kinase (IKK) and degradation of I- κ B- α [65, 66]. Hsp70 may also facilitate the elimination of DNA-damaged cells [67]. Inhibitor of growth (ING) proteins acts as tumor suppressors; their expression is down-regulated in human cancers that transmit a death signal and bind to histones and therefore control chromatin remodeling and p53 activity [68, 69]. These proteins boost the function of Hsp70, which in turn induces TNF- α receptor-mediated apoptosis by preventing IKK activity and blocking of NF- κ B survival pathways [68]. In addition, Hsp70 coupled with Hsp40 inhibits Bax translocation and thereby prevents permeabilization of mitochondrial membranes

and the subsequent release of cytochrome c and apoptosis-inducing factor (AIF) [70]. This function of Hsp70 depends on a chaperone and ATP hydrolysis activity [71].

Hsp70 also acts at the post-mitochondrial level, blocking apoptosis downstream to cytochrome c and upstream to caspase-3 [72]. It inhibits formation of the apoptosome by interaction with its ATPase domain [73, 74]. Furthermore, in TNF- α -mediated apoptosis, Hsp70 prevents the characteristic morphological changes of dying cells but does not preclude caspase-3 activation [75]. Activated caspase-3 leads to activation of caspase-activated DNase (CAD), which is responsible for DNA degradation during apoptosis. Hsp70 with its co-chaperones, Hsp40 and inhibitor of CAD (ICAD), regulate enzymatic function and proper folding of CAD. ICAD, with the Hsp70-Hsp40 complex, recognizes and binds to the transit state of CAD and increases its activity in T-cell receptor-mediated T-cells [76]. An early target of caspase-3, poly (ADP-ribose) polymerase (PARP), is necessary to prevent necrosis and inflammation during apoptosis [77, 78]. In the nuclei of cells undergoing single-strand DNA breaks after heat treatment, Hsp70 interacts with PARP1, XRCC1, and other DNA repair proteins [77, 78]. These findings suggest that Hsp70 restores DNA integrity through formation of the protein repair complex. Caspase-3 targets a transcription factor, GATA-1; however, Hsp70 accumulation in nucleus protects GATA-1 from caspase-3 cleavage and thereby increases erythroid cell differentiation and survival [10].

Hsp70 and the extrinsic/death receptor pathway

Hsp70 blocks TNF- α -mediated apoptosis; however, it fails to protect Bid-homozygous knockdown in MEF cells [79]. In primary human fibroblast cells, Hsp70 inhibits Bid cleavage through activation of caspase-8/10 [79]. TNF- α mediated stimulation of hematopoietic cells induces activation of pro-apoptotic, double-stranded RNA-dependent protein kinase (PKR) [80]. In TNF- α -induced apoptosis, Hsp70 interacts with the FANCC protein (Fanconianemia complementation group C, an inhibitor of PKR) via its ATPase domain and forms a ternary complex with FANCC and PKR [80, 81]. It also resists TRAIL-induced apoptosis and formation of a death-inducing signaling complex with death receptors DR4 and DR5 [38]. The function of Hsp70 in Fas-induced apoptosis is poorly understood; however, adverse effects depend on the cell context [82, 83].

Hsp70 and the caspase-independent apoptotic pathway

Activation of the intrinsic pathway, triggered by the release of cytochrome c, induces AIF and translocation of endonuclease G (Endo-G) to the nucleus, where it induces caspase-independent nuclear changes [84]. In cells with Apaf-1 and caspase-9 knockdown, Hsp70 inhibits the caspase-independent apoptotic pathway, suggesting that it also prevents cell death in Apaf-1- or caspase-9-knockdown cells incubated with or without a caspase activator/inhibitor. These findings indicate that the cytochrome c/Apaf-1/caspase pathway is not the sole pathway for Hsp70 interactions [85, 86]. In cell-free systems, Hsp70 binds to AIF and inhibits AIF-mediated chromatin condensation, a mechanism by which it could protect cells from AIF-induced apoptosis. Thus, endogenous Hsp70 controls AIF-mediated apoptosis; in Jurkat T cells, down-regulation of Hsp70 sensitizes cells to serum withdrawal and AIF release [86]. Further, in Apaf-1^{-/-} cells, Hsp70 inhibits erythroblast apoptosis by blocking nuclear import of AIF [87]. Hsp70 with Endo-G protects against DNA fragmentation; this association could use AIF as a molecular bridge [88]. Hsp70 resides in the endolysosomal membranes of tumor cells and stressed cells; in HeLa cells, it prevents the release of lysosomal cathepsin into the cytosol (Fig. 3) [89, 90]. Hsp70-positive lysosomes show increased size and resistance to chemical and physical membrane destabilization [91]. In murine fibroblasts, Hsp70 also protects cells from UV-A- and UV-B-induced apoptosis. This protection is mediated through inhibition of IL-6 release, which is induced by UV light (Fig. 3) [91].

Hsp70: a protein associated with tumorigenicity

Under non-stressed conditions, cells express Hsp70 at basal levels. Enhanced expression, a characteristic of cancerous or stressed cells, increases survival of these cells. Further, anti-cancer therapy elicits Hsp70 expression, which has a cytoprotective effect. Clinical studies indicate that Hsp70 predicts for a poor prognosis because malignant cells express more Hsp70 during tumor progression (endometrial cancers, osteosarcomas, and renal cell tumors) as compared to normal cells [92]. Hsp70 and prostate-specific antigen are markers used to identify patients in early stages of prostate cancer [93]. Further, Hsp70 is abundantly expressed during the progression of chronic myeloid leukemia [94]. In HL-60/BCR-ABL and K562 cells, increased expression of Hsp70 helps cells resist imatinib-mediated cell death (imatinib, a chemotherapeutic agent used to block Bcr-Abl tyrosine kinase activity) [95]. In gastric epithelial cells, the expression of Hsp70 is

elevated after infection with *Helicobacter pylori* [95]. In addition, Hsp70.2, a member of the Hsp70 family, is highly expressed during spermatogenesis and breast cancer progression, thereby delaying senescence [95, 96]. Enhanced expression of Hsp70 is associated with tumorigenesis for breast cancer, endometrial cancer, gastric cancer, and acute leukemia; with poor prognoses; and with resistance to chemo-and radiation therapy [13, 97-100]. Nuclear accumulation of Hsp70 is a diagnostic marker for epithelial dysplasia, and antibodies against Hsp70 are present in sera of patients with hepatocellular carcinoma [101, 102].

Please insert Table 1 here

Table 1. Demonstrates specific cancers and their association with Hsp70

	Cancer Type	Effects of Hsp70 expression	Ref.
1.	Leukemia, MCF-7 breast cancer	High Hsp70 expression increase cancer growth and survival	[103, 104]
2.	Gastric, endometrial cancer	Hsp70 silencing with RNAi inhibits human gastric cancer growth and induces apoptosis	[105, 106]
3.	Gastric cancer	High Hsp70 expression induced cancer survival	[107, 108]
4.	Colon and lung cancer	High Hsp70 expression was associated with overall survival High Hsp70 plays cytoprotective role in cancer	[109-112]
5.	Breast & Gastric cancer	Hsp70 showed anticancer effect	[113-115]
6.	Prostate cancer	High Hsp70 contributes in prostate cancer development	[109, 116]

Hsp70, which inhibits apoptosis upstream and downstream to mitochondria, is a promising therapeutic target for lowering drug resistance in cancer cells [38, 94]. Stress-mediated expression of Hsp70 promotes tumorigenesis in cancer cells (colon cancer, melanoma, and pancreatic adenocarcinoma); its down-regulation is associated with decreased tumorigenicity [44, 45, 117-119]. In accord with this, a Hsp70 antisense construct was used to kill cancer cells in the absence of additional stimuli [120]. Down-regulation of Hsp70 is cytotoxic to transformed cells; however, it is undetectable in non-transformed cells [118, 121]. Therefore, Hsp70 knockdown sensitizes or kills cancer cells preferentially to normal cells. The constitutively stressed phenotype of cancer cells depends on the cytoprotective function of Hsp70 (Fig. 3).

Hsp70: cancer treatment

Hsp70 is a druggable target in comparison to other HSPs because they are regulated by nucleotides [122, 123]. Hsp70/Hsp90 is the only ATPase that is regulated by the inhibition of its ATPase activity [122, 123]. Therefore, targeting of Hsp70 is an attractive strategy for cancer treatment. Although several inhibitors have been designed for Hsp90, and some of these are in clinical trials, few are known for Hsp70. Gene transcription of Hsp70 is regulated by the transcription factor, HSF1, which becomes activated in response to stress stimuli [124]. Since activated HSF1 induces Hsp70 expression [124], inhibition of HSF1 could be an effective approach to block the expression of Hsp70. Inhibition of HSF1 activation can be achieved by the flavonoid, quercetin; by diterpenetriperoxide; and by triptolide. These compounds, along with benzopyrene, inhibit the expression of Hsp70; other HSPs may remain unaffected [43, 125-129]. In K562 cells, resveratrol, a non-specific inhibitor of Hsp70, inhibits Hsp70 expression by blocking Akt-kinase activity and up-regulating ERK1/2 kinase activity [130].

Please insert Fig. 4 here

AIF-derived peptides (150-228aa) targeting Hsp70 sensitize cancer cells to apoptosis [131]. These peptides carry the AIF regions (150aa-228aa) required for Hsp70 binding in its PBD but lack the pro-apoptotic function of AIF. One of these inhibitors, ADD70 (AIF-derived decoy for Hsp70) decreases tumor sizes in rat colon cancers and melanomas (B16F10) and sensitizes these cancers to cisplatin. ADD70 shows anti-tumor effects in syngeneic animals but not in immunodeficient mice; it also increases tumor-infiltrating cytotoxic CD8⁺ T-cells [121].

In lymphoma cells, interaction of a small molecule (HS-72) with Hsp70 leads to aggregation of misfolded proteins and destabilization of lysosome membranes, thus inducing autophagic cell death [132]. 2-Phenylacetylene sulfonamide interacts with the C-terminal of Hsp70 and inhibits its expression [133]. In B-CCL cells, however, PES induces the caspase-dependent apoptotic pathway [134]. Hsp90 inhibitors that displace ATP from Hsp70 may target Hsp70. Although further investigations are needed to explore the underlying mechanisms, some results are encouraging [135]. The adenosine-derived compound, VEK-155008, targets the ATPase domain of Hsp70/hsc70 and blocks its chaperone activity; it also induces death of colon HCT116 carcinoma cells [136]. To date, however, no evidence derived with intact animals is available.

Although azure C, methylene blue, and myricetin are potent inhibitors of human Hsp70, their specificity for tumor-derived Hsp70 remains to be addressed [137].

Please insert Table 2 here

Table 2. Demonstrates various Hsp70 inhibitors including their sites of action and applications in pre-clinical and clinical trials. Dotted lines show that the given compound may or may not be beneficial in clinical trials.

	Hsp70 inhibitors	Site of interaction	Tested in Clinical trials	Ref.
1.	MKT-077	N-terminal ATP binding domain	Yes	[122,123]
2.	Dihydropyrimidines	N-terminal ATP binding domain		[122, 123, 137]
	i) SW02	N-terminal ATP binding domain	Yes	[124,125]
	ii) MAL2-IIB	N-terminal ATP binding domain	Yes	
	iii) MAL3-101	N-terminal ATP binding domain	Yes	
	iv)NSC630668-R/I	N-terminal ATP binding domain		
3.	Sulfoglycolipids	N-terminal ATP binding domain		[122, 123, 138, 139]
	i) Sulfogalactoglycerolipid	N-terminal ATP binding domain		
	ii) Sulfogalactosylceramide	N-terminal ATP binding domain		
	iii) adamantylSGC	N-terminal ATP binding domain		
4.	Flavonoids	N-terminal ATP binding domain		[122, 123, 137, 140]
	i) Epigallocatechin	N-terminal ATP binding domain	Yes	
	ii) Myricetin	N-terminal ATP binding domain		
5.	Apoptozole	N-terminal ATP binding domain		[122, 123, 141]
6.	VEK-155008	N-terminal ATP binding domain		[122]
7.	Aptamer A17	N-terminal ATP binding domain		[122, 123]
8.	Dibenzyl-8-aminoadenosine analog	N-terminal ATP binding domain		[122, 123]
9.	cmHsp70.1mAb	Interact with Hsp70 epitope	Yes	[122,

				123, 142]
10.	PES	C-terminal/peptide binding domain	Yes	[122, 123]
11.	Pyrrhocoricin	C-terminal/peptide binding domain		[122, 123, 143]
12.	Geranylgeranylacetone	C-terminal/peptide binding domain	Yes	[122, 123, 144]
13.	Fatty acid acyl benzamides	C-terminal/peptide binding domain		[122, 123, 145]
14.	Pifichrin-μ	C-terminal/peptide binding domain		[122, 123, 132]
15.	aptamerA8	C-terminal/peptide binding domain		[122, 123]

MKT-077, a cationic rhodacyanine dye, acts upon ABD-Hsp70 in cancer cells. This compound, which migrates to mitochondria and inhibits mitochondrial Hsp70, is being tested in Phase-I clinical trials as an anticancer agent [146]. Although MKT-077 does not interact with Hsp70, it deserves further investigation due to its drug-like nature [147]. NSC 630668, a dihydropyrimidine, and a second-generation compound, MAL3-101, inhibit the ATPase activity of Hsp70 and the proliferation of SK-BK-3 cancer cells [148]. MAL2-11B, an inhibitor of polyomavirus, blocks the activity of the viral J-domain protein and T-antigen; further investigations are needed to dissect this process [149]. The combination of peptide aptamers consisting of an *E. coli* thioredoxin scaffold shows (8aa or 13aa) peptide loops that block Hsp70 expression by interacting with the ATP-binding domain of Hsp70, as determined in yeast 2-hybrid systems. Among these, A17 induces apoptosis in tumors in response to anti-cancer drugs. It inhibits the chaperone activity of Hsp70 but has no effect on Hsp70/Hsp90 [131].

Several synthetic compounds disrupt the interaction between Hsp70 and its co-chaperones. Active in this regard are pyrimidotriazinediones, a new class of drugs that interact with Hop/Hsp70 and are toxic to WST-1 cells [150]. Drugs targeting huntingtin-interacting protein 1 block Hsp70-chaperone activity and stimulate neurodegeneration [151].

Targeting Hsp70 is a new therapeutic approach; most compounds active in this regard are Hsp90/Hsp70 inhibitors that induce apoptosis in cancer cells [46, 121]. Various HSP inhibitors are being evaluated in clinical trials. In treated patients, however, there is enhanced expression of Hsp70 in cancer cells, which is not a positive sign, for Hsp70 accumulation reduces the possibility of cell death, thus decreasing the anti-tumor efficacy of Hsp70 inhibitors. Treatment

with chemotherapeutic drugs increases the expression of Hsp70 and induces TGF- β -signaling [152]. Hsp70 knockdown by siRNA increases sensitivity of cancer cells to tanespimycin (17-N-allylamino-17-demethoxygeldanamycin, 17-AAG) [153]. However, in HCT116 cells, knockdown of both Hsp70 and Hsc70 induces proteosomal-dependent degradation of Hsp90 client proteins and apoptosis [154]. In addition, a combination of an adenosine-derived inhibitor of Hsp70, VER-155008, and 17-AAG induces apoptosis in HCT116 colon carcinoma cells [136]. Furthermore, the anti-cancer activity of 17-AAG is elevated in colon cancer cells with blocked Hsp70 [121].

Inhibitors of Hsp70 and Hsp90 combined with histone deacetylase inhibitors increase the cell surface expression of Hsp70 on hematopoietic cancer cells [155]. An early response of extracellular Hsp70 is to activate suppressive myeloid immune cells [156]. Therefore, Hsp70 and Hsp90 inhibitors should be evaluated for a synergistic effect in an experimental model (Fig. 4). Chelerythrine, a benzophenanthridine alkaloid and a specific inhibitor of protein kinase C (PKC), downregulates the expression of Hsp70 in Dalton's lymphoma cells [157, 158]. Staurosporine, a non-specific inhibitor of PKC, also shows a similar effect [158].

Hsp70: Immunological function

In addition, to cytoprotection and chaperone activity, tumor-derived Hsp70 has immunomodulatory function(s) that activate the immune system. The immunogenic function of tumor derived/exogenous Hsp70 is exerted through its antigenic peptides [159].

In humans, the tumor burden enhances the expression of serum Hsp70, and tumor cells are the natural reservoir of Hsp70. Hsp70 is abundantly expressed on tumor cell surfaces during tumor progression; however, treatment with interferon- γ induces the release of Hsc70 from tumor cells [160, 161]. Furthermore, members of the Hsp70 family often infiltrate antigen-presenting cells (APCs) and are present in neuroblastomas and lung and colon adenocarcinomas [136, 162].

Please insert Fig. 5 here

Cytosolic Hsp70 is transported to the plasma membrane in association with other proteins that possess a transmembrane domain. However, lack of a leader peptide does not allow Hsp70 to localize on the membrane in that manner. A possibility is that Hsp70 interacts with lipids in/on the membrane. In PC12 tumor cells, Hsp70 interacts with phosphatidylserine (PS) and shows co-

localization with PS in the outer membrane leaflet, which is facilitated by a P-flipping mechanism [163]. There are two possible mechanisms by which Hsp70 is released from cells: a passive mechanism that results from necrosis, trauma, or surgery, and/or an active mechanism by which non-classical proteins are released from immunologically potent exosomes [164, 165]. In HepG2 cells, Hsp70 is released from the extracellular environment after heat shock. Membrane-bound Hsp70 acts as an activator of macrophages, free recombinant Hsp70 does not [166].

Hsp70 shows immunogenicity that activates both arms of the immune system (innate and adaptive immune responses) [167, 168]. In a receptor-mediated manner, endocytosis of Hsp70 stimulates the MHC-I presentation pathway of professional APCs and cytotoxic T-lymphocytes [72]. Thus, tumor-derived Hsp70 can be used as a tumor-specific vaccine [169]. Hsp70 also induces the release of pro-inflammatory cytokines from innate immune cells, thereby increasing the expression of co-stimulatory molecules [162, 170]. Furthermore, Hsp70 activates the lytic machinery of natural killer (NK) cells against tumors expressing Hsp70 on the cell surface [171]. These characteristics of Hsp70 have led to the view that Hsp70 acts as an endogenous adjuvant and immunological danger signal [172].

Hsp70 and Hsp90 are regulators of the immune system. Hsp70 elicits anti-cancer immune responses, but Hsc70 does not [173]. Therefore, Hsp70 is the foundation of immunogenicity; following cross presentation of tumor-derived Hsp70 on MHC-I molecules, a T-cell response is generated [174-181]. Hsp70 enters into the endogenous antigen-processing pathway, primes CD8⁺ T cells for antigen production, and becomes involved in cross presentation [181-184]. Tumor-derived Hsp70 is endocytosed by APCs with the help of HSP receptors (CD91, CD40, TLR2/4 + CD14, CD35, Lox-1, and SR-A) and presented on the MHC-I molecule, thereby producing a CD8⁺ T-cell response against cancerous stimuli. Recombinant Hsp70 induces cross presentation via the formation of various complexes and the uptake of antigens. However, recombinant Hsp70 does not stimulate innate immune responses in dendritic or B cells [185]. However, tumor-derived Hsp70 enhances MHC-II restricted peptide presentation and CD4⁺ T-cell activation [186]. In the absence of immunogenic antigens, Hsp70 gives danger signals for the immune system [187]. Therefore, Hsp70 apparently inhibits tumor growth via two pathways: one antigen-dependent and the other antigen-independent. The C-terminal domain of Hsp70 typically produces an antigen-independent response, which includes stimulation of NK cells against tumor challenges [63]. Treatment with Hsp70 results in a stronger anti-tumor response

[188]. Hsp70 also affects cytokines. In APCs, Hsp70 induces the release of pro-inflammatory cytokines, including IL-1 β , IL-6, and TNF- α [63]. In established melanomas, Hsp70 acts as an immune adjuvant that induces TNF- α production [189]. The role of Hsp70 in cytokine production has been studied (Fig. 5) [53, 159, 190].

Hsp70: an immunotherapeutic drug

On the basis of its immunological function, Hsp70 can be used as an immunotherapeutic drug because its potent adjuvant nature induces cancer autoimmunity [191]. Hsp70 derived from A-20 leukemic cells acts as an antigen and induces the production of an A-20-specific antibody, resulting in complement-dependent cytotoxicity against these cells [192]. Further, dendritic cells pulsed with Hsp70 induce immunity against B-16 melanoma, which increases the therapeutic value of a Cox-2 inhibitor [193]. The C-terminus of Hsp70 interacts with CD8⁺ T and CD4⁺ T cell epitopes and enhances tumor immunity beyond the effect of the CD8⁺ T cell epitope alone in eliminating tumor cells [159, 194]. In experimental models, a similar interaction with HPV-16 E7 induces an antigen-specific cytotoxic T-cell response [195]. C-terminal interaction of Hsp70 with a recombinant N-domain calreticulin/E7 generates an antitumor immune response [195]. Although some HSP-based vaccines are currently being used in clinical practice, an improved formulation of these vaccines may be derived by extraction and use of the Hsp70 complex from dendritic tumor-fused cells [141]. Hsp70-based vaccines derived from the fusions of dendritic and tumor cells reverse immunotolerance of cancers more efficiently than vaccines derived from tumor cells alone [196]. Autologous anti-tumor vaccines prepared with hydroxyapatite particles and Hsp70/27/Grp96 can be used safely [197, 198]. NK cells, which are effectors of innate immune responses, are involved in anti-tumor immunity produced by vaccinations with chaperone-rich cell lysates [199]. Hsp70 induces activation of NK cells [200]. A14-mer peptide (TKD), derived from the C-terminal of Hsp70 (PBD) 450aa-463aa, shows similar immunostimulatory capabilities on NK-cells as full-length Hsp70 [201, 202]. NK cells incubated with a cytokine plus soluble Hsp70 or the TKD peptide enhance expression of the activating receptor (CD14) and increase migration capacity [200]. Hsp70, present on tumor cell surfaces but not on surfaces of normal cells, is considered as a tumor-selective target. In a mouse model, peripheral blood lymphocytes incubated with TKD-peptide and IL-2 induce migration of NK cells towards Hsp70 membrane-positive tumor cells [203]. An Hsp70-based vaccine is in phase-I

clinical trials; an advantage of this approach is the excellent safety and the bioavailability of the synthetic Hsp70 peptide that stimulates NK-cells [204].

Conclusions

Hsp70 is the most ancient anti-stress defensive system that promotes tumor cell survival by interacting at several points in apoptotic signaling pathway(s) [109-112]. Researchers are attempting to improve modern cancer treatment therapies by implementing Hsp70 inhibitors for the development of novel drugs in the near future [113-115]. Hsp70 demonstrates various functions, including acting as a cellular lifeguard and exerting anti-apoptotic effects. It is also involved in modulation of intrinsic and extrinsic apoptotic signaling and, in cancer cells, modulates caspase-independent apoptosis. Since Hsp70 has anti-cancer and immunogenic properties, there is a search for Hsp70 inhibitors. However, preclinical and clinical evaluations have not yet been accomplished, unlike Hsp90 inhibitors, which are already in phase II/III clinical trials. Nevertheless, clinical trial results obtained to date are not favorable. Such results may be due to enhanced expression of Hsp70 in tumors. Extracellular Hsp70 is believed to be immunogenic because it acts as an adjuvant and, combined with chaperone(s), it may prove useful for vaccine production and cancer treatment. Increased expression of extracellular Hsp70 is a sign of a poor prognosis for cancer. In syngeneic mice, inhibition or depletion of Hsp70 causes tumor regression, and Hsp70 inhibition results in an anti-tumor immune response and apoptosis of the target cells. Therefore, targeting Hsp70 is a promising therapy for cancer patients.

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Figure Legends

Fig. 1 Structure of Hsp70 with sites of action [22-24]. N-terminal shows ATPase domain with associated co-chaperones (Bag1/Hip/hsp110 or hspBP1). However, C-terminal represents EEVD domain with its co-chaperone (Hop/Hdj-1/Chip) and peptide binding domain. J-domain localizes in the central approximately.

Fig. 2 Diagrammatic presentation of the chaperone functions of Hsp70 [22-25]. Hsp70 binds to unfolded protein in the presence of J-proteins using ATP. ATP hydrolysis stimulates J-protein release that further led to release of correctly folded protein(s) and Hsp70 become available for next cycle.

Fig. 3 Schematic representation of the function of Hsp70 in the apoptotic signaling and survival pathways [205-208]. SAPKs stimulate synthesis of Hsp70 that leads to cell survival. High Hsp70 interferes at several levels in the apoptotic signaling such as apoptosome, caspases, and cathepsins. Hsp70 also contributes in heterodimerization of bcl2 and Bcl-xL that block Bax heterodimerization.

Fig. 4 Schematic representation of the inhibition of Hsp70 in cell survival pathways [159, 181, 209]. Hsp70 can be inhibited at transcription and post-transcription levels as indicated in the figure. Inhibition of Hsp70 may results in induction of cell death.

Fig. 5 Diagram depicting involvement of Hsp 70 in cross presentation [159, 181, 209]. Receptor mediated endocytosis of Hsp70 was presented by MHC-II in association with CD4⁺ T cells. However, small fragments of Hsp70 was presented by MHC-I molecule in association with CD8⁺ T cells, called “cross presentation”.

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Figure 1

Figure 2

Figure 3

Figure 4

Figure 5