Review

Immunotherapy

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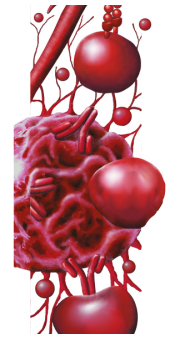

Heat-shock proteins in diagnosis and treatment: an overview of different biochemical and immunological functions

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Heat-shock proteins (HSPs) have been involved in different functions including chaperone activity, protein folding, apoptosis, autophagy and immunity. The HSP families have powerful effects on the stimulation of innate immune responses through Toll-like receptors and scavenger receptors. Moreover, HSP-mediated phagocytosis directly enhances the processing and presentation of internalized antigens via the endocytic pathway in adaptive immune system. These properties of HSPs have been used for development of prophylactic and therapeutic vaccines against infectious and noninfectious diseases. Several studies also demonstrated the relationship between HSPs and drug resistance as well as their use as a novel biomarker for detecting tumors in patients. The present review describes different roles of HSPs in biology and medicine especially biochemical and immunological aspects.

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Organisms respond to environmental, metabolic or pathophysiological stress conditions (e.g., high temperature, decreased oxygen supply, infectious agents, toxic substances or inflammatory mediators) using selectively upregulation of the heat-shock proteins (Hsps) [1]. These proteins have been classified into six major families based on their molecular masses such as HspH (Hsp110), HspC (Hsp90), HspA (Hsp70), DNAJ (Hsp40), HspB (small HSPs) and the chaperonin families HspD/E (Hsp60/Hsp10) and CCT (TRiC) [2,3]. On the other hand, HSPs are often divided as the constitutive (cognate) and inducible forms. For instance, the constitutively expressed Hsp73 and stress-inducible Hsp72 were shown to be highly related proteins. Both Hsp72 and Hsp73 could act as molecular chaperones and thus interact transiently with nascent polypeptides [4]. Another report indicated that distribution of the constitutive and inducible HSP70, Hsc70 and Hsp70, in different parts of the rat eye were related to the metabolic needs for absorption and detection of light. Hsc70 is constitutively expressed in mammalian cells, whereas Hsp70 is highly induced in cells under stress conditions [5]. Generally, Hsps repair damaged proteins and increase cell survival, through chaperone function [6]. The inducible Hsp expression is regulated by four heat-shock transcription factors (HSFs) in mammalian cells [7]. At first, trimerization of the HSFs occurs before binding to the heat-shock element (HSE) in DNA. The process of activation and DNA binding of HSF1 is also controlled by its stress-dependent phosphorylation. Moreover, a small nuclear HSBP1 protein could strongly bind to HSF1 monomers suppressing HSF1 activation [8]. On the other hand, high expression of Hsp70 led to convert the active HSF trimer to inactive HSF monomer, a key point in the reduction of the heat-shock transcriptional response. The reports showed that under normal conditions, HSF1 is held in an inactive complex with Hsp90 chaperone. Under cell stress or blocking Hsp90, HSF1 is activated, released from HSF1–Hsp90 complex, trimerized and translocated to the nucleus [9,10]. The studies indicated that most mitochondrial and chloroplast proteins (~95%) are encoded by nuclear genes transferred to the cytosol as precursor proteins and then imported into the organelle by specific protein import machinery including the ATP-driven cytosolic HSP70 family (cpHSP70) [11]. A study also showed that Hsp20 is essential for protein kinase D-1 nuclear translocation [12]. The Hsps have been involved in various

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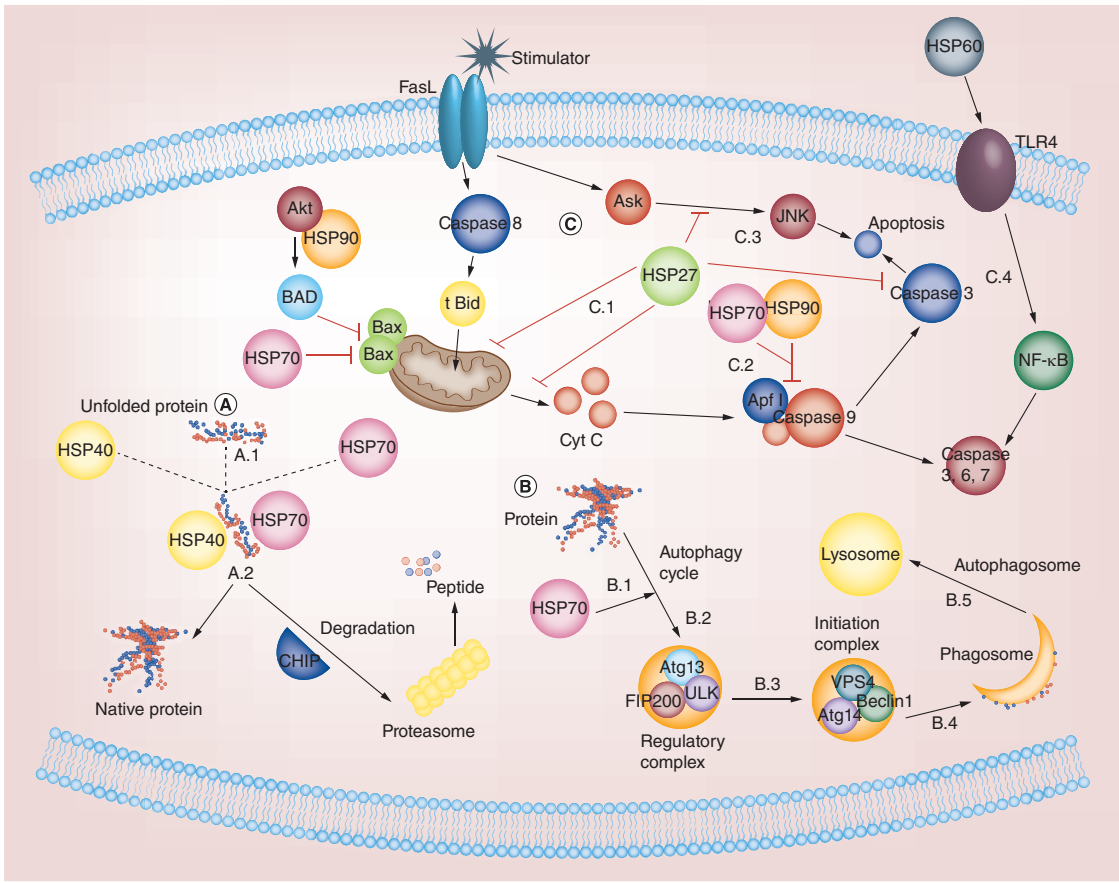

Figure 1. Biochemical functions of heat-shock proteins. (A) Chaperone activity: accumulated misfolded proteins bind to HSPs for the maintenance of protein hemostasis **(A.1)**. For instance, Hsp70 interacts with Hsp40 for converting misfolded proteins to native form and/or these chaperones guide misfolded proteins to proteasome for degradation into small peptides **(A.2)**. **(B)** Autophagy pathway: At first, Hsp70 binds to the protein and autophagy cycle begins **(B.1)**. The regulatory complex contains ULK1, Atg13 and FIP200 activated by Hsps **(B.2)**. Then, the initiation complex composed of Beclin1, VPS4 and Atg14 are stabilized by Hsps **(B.3)**. After that, phagosome is formed by collecting phagosomal markers **(B.4)**. Finally, the completed autophagosome fuses with a lysosome **(B.5)**. **(C)** Apoptosis pathway: The proapoptotic signals through FasL can be modulated by Hsps at the mitochondrial and postmitochondrial levels. Hsp27 and Hsp70 can inhibit the release of proapoptotic proteins from mitochondria through suppression of active truncated Bid (tBid) and Bax, respectively **(C.1)**. Sequestering cytosolic cytochrome C by Hsp27, Hsp70 and Hsp90 from apoptosis protease-activating factor (Apaf-1) resulting in the inhibition of apoptosome formation and thus prevention of apoptosis activation **(C.2)**. Hsp27 can also suppress the apoptosis through the inactivation of apoptosis-signal-regulating kinase (Ask) and caspase 3 **(C.3)**. Also, interaction of Hsp60 and TLR4 can mediate NF-κB signaling pathway resulting in the activation of caspase 3, 6, 7 and DNase **(C.4)**.
HSP: Heat-shock protein.

functions including chaperone activity, protein folding, apoptosis, autophagy and immunity. Generally, Hsps were studied for their role in protecting cells from high temperature and stress conditions. Moreover, several roles for Hsps in the immune system were determined including intracellular roles in antigen presentation and expression of innate receptors, and extracellular roles in tumor immunosurveillance and autoimmunity [13, 14]. Extracellular Hsps could exert potent effects on the immune response playing both stimulatory and regulatory roles [15]. The studies showed that Hsps have a dual function depending on their intracellular or extracellular location. Intracellular Hsps have a cytoprotective function with different mechanisms. Several Hsps such as Hsp70 or Hsp27 could directly interact with various components of the programmed cell death machinery. On the other hand, extracellular located or membrane-bound Hsps mediated immunological functions by modulation of the adaptive or innate immune systems [16]. In this review, each role of Hsps will be described in detail. Figures 1 & 2 briefly show the biological functions of Hsps.

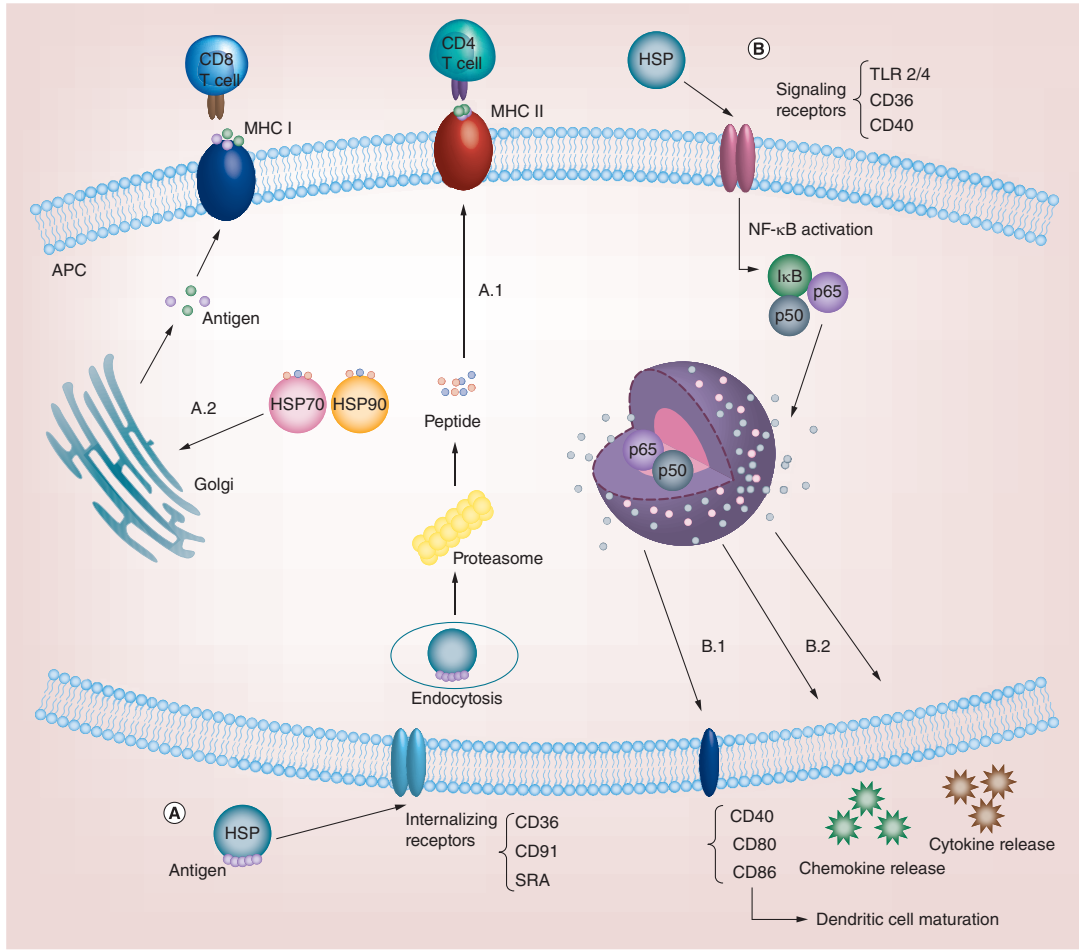

Figure 2. Immunological functions of heat-shock proteins. (A) Adaptive immune activity: the binding of Hsp–antigen complex to the internalizing receptors on the antigen-presenting cells can lead to endocytosis. The proteasome-processed peptides can be directly presented on the MHC-II **(A.1)** or followed by cross-presentation on the MHC class I through Golgi **(A.2). (B)** Innate immune activity: Hsps can increase the costimulatory molecules through the induction of cell-signaling receptors. Hsps engage signaling receptors that trigger NF-κB activation leading to upregulation of the expression of costimulatory molecules (e.g., CD40, CD80 and CD86) **(B.1)**, and increase the secretion of cytokines (e.g., IL-12, TNF-α and GM-CSF) and chemokines (e.g., RANTES and MCP-1) **(B.2).**
HSP: Heat-shock protein.

HSP structure

The most important HSP families contain HSP70, HSP60, HSP90 and small HSP (sHSP). Their structures are different. Briefly, the structure of Hsp70 includes an N-terminal ATPase domain (~44 kDa), a substrate binding domain (~18 kDa) and a C-terminal domain (~10 kDa) [17]. The conserved structure of cytosolic Hsp90 has an N-terminal domain containing the cochaperone-binding motif and an ATP and drug-binding site (e.g., geldanamycin and radicicol), an intermediate domain for binding to cochaperone and client proteins, and a C-terminal domain including a dimerization motif, a second drug-binding site and a conserved MEEVD pentapeptide [18]. Most information on structure of Hsp60 is available from its prokaryotic homolog (i.e., GroEL). GroEL protein is composed of 14 identical subunits of 57 kDa, each forming two heptameric rings arranged back-to-back. Each GroEL monomer possesses three structural domains including apical, intermediate and equatorial (E1 and E2 subdomains) [19]. The members of sHSP family have low molecular masses (~13–43 kDa) and contain a large N-terminal domain, a conservative α-crystallin domain (~90 residues) and a short C-terminal extension. The α-crystallin domain forms stable dimers that are the building blocks of the large sHsp oligomers [20]. Supplementary 1 represents the structures and properties of major HSPs.

Chaperone activity: protein folding, proteostasis & protein turnover

The proteins are slightly stable in their physiological environment and thus susceptible to misfolding and aggregation [21]. For maintaining protein function and cell homeostasis, molecular chaperones are required especially under stress conditions. The molecular chaperones are a large family of proteins that lead to assemble certain polypeptide chains correctly. Besides their major role in protein folding, chaperones are involved in protein transport and degradation, dissociation of aggregate and refolding of stress-denatured proteins, and assembly of macromolecular complex. Indeed, many of molecular chaperones are among the HSPs selectively expressed in cells exposed to metabolic stress [21]. Indeed, molecular chaperones as the protein homeostasis machinery were involved as the first line of defense against the diseases such as cardiovascular diseases, alcoholic hepatitis, cataract, cystic fibrosis, phenylketonuria suggesting them as a molecular marker for diagnostics. The experiments showed that overexpression of chaperones in mice were effective in suppressing neurodegeneration [22–24]. Figure 1A shows chaperone activity of HSPs.

Protein folding

Protein folding is the process that a protein structure obtains its functional shape or conformation. The protein folding requires several molecular chaperones that form transient complexes with their protein substrates through hydrophobic interactions. For example, the Clp/HSP100 chaperones cooperated with the Hsp70 chaperones in refolding and reactivation of thermal-aggregated proteins [25]. Also, Hsp78, a member of the Clp/Hsp100 family localized in the mitochondria of *Saccharomyces cerevisiae* along with the mitochondrial Hsp70 was effective in the refolding of heat-inactivated proteins [26]. In the mammalian system, the molecular chaperones Hsp70 and Hsp90 formed a multichaperone complex and were connected by Hop cochaperone involved in the folding and maturation of key regulatory proteins (e.g., steroid hormone receptors, transcription factors and kinases) [27]. As known, there are several cochaperones that help protein folding and other functions of Hsp chaperones. For instance, p23 was a cochaperone for Hsp90 that participated in the folding of different cell regulatory proteins [28]. Cdc37 was also other Hsp90 cochaperone. The findings indicated that phosphorylation of Cdc37 by casein kinase II could regulate the ATP-driven reaction cycle of Hsp90 and Cdc37 with protein kinases [29]. Moreover, the hepatitis B virus (HBV) X-associated protein-2 was the Hsp90 cochaperone that inhibited importin binding to the mouse aryl hydrocarbon receptor, a ligand-activated transcription factor and ligand-independent nucleocytoplasmic shuttling of the mouse aryl hydrocarbon receptor [30]. On the other hand, $P58^{IPK}$ was a member of the J-domain protein family and a cochaperone stimulating the ATPase activity of Hsc70 in a protein-refolding system [31]. The polypeptide-binding domain of the chaperone Hsp70 stimulating its ATPase activity was regulated by cochaperones such as the Bag domain nucleotide exchange factors, as well [32]. AtTDX, a thioredoxin-like *Arabidopsis* protein was known as a novel component of the Hsp70 chaperone system. The N-terminal fragment of AtTDX was related to the cochaperone Hsp70-interacting protein HIP, whereas its C-terminal fragment included a thioredoxin domain. The HIP domain of AtTDX was capable of interacting with the ATPase domain of yeast Hsp70 chaperone [33]. The studies demonstrated that Hsp40 was also an important family of cochaperones that plays a key role in regulating Hsp70 interaction with client proteins [18].

Protein turnover

Protein turnover is the balance between protein synthesis and protein degradation. As known, stress conditions can cause protein misfolding and aggregation. In humans, protein aggregation leads to several diseases such as Alzheimer, Huntington, Parkinson, Creutzfeldt-Jakob, diabetes (Type II), aging and cancer. Different classes of Hsps are involved in control of protein quality. For instance, Hsp70-targeted proteins for degradation when the protein could not be correctly renatured as well as folding nascent proteins, and refolding denatured proteins [34]. Indeed, the cochaperones Bag-1 and CHIP helped Hsp70 for degradation of unfolded or misfolded proteins. CHIP possesses ubiquitin ligase E3 activity for unfolded proteins directed toward the proteasom pathway (Figure 1A [35]). On the other hand, the Hsp70/Hsp40 cooperative system could degrade small aggregates alone but larger aggregates required Hsp104, an AAA-ATPase chaperone of the HSP110 family, as well. In fact, Hsp104 was responsible for the disaggregation of denatured proteins and thus could directly act in the protein turnover process. Also, Hsp90 showed various roles in the conformational regulation of several signaling proteins. The role of Hsp90 in cancer was related to Hsp90 client proteins including FLT3, Bcr-Abl and ErbB2. Hsp90 inhibition could stimulate apoptotic cell death associated with the cytosolic accumulation of cytochrome c and SMAC/Diablo in mitochondrial pathways [34,36].

HSPs in apoptosis

Apoptosis is a form of programmed cell death that occurs in multicellular organisms. The conserved proteolytic enzymes so-called as caspases have crucial functions in apoptosis accompanied by different molecular and cellular changes, especially decreased cellular volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and ingestion by phagocytes. The caspases were classified into various groups based on their roles including initiator and effector caspases. Generally, the initiator caspases (e.g., caspase-8 and caspase-9) activated by upstream signals (i.e., TNF-α, Fas, starvation and cellular dysfunction) can activate the effector caspases (e.g., caspase-3 and caspase-7) leading to mitochondrial release of cytochrome c [37]. The HSPs increased resistance to cell death induced by various conditions. For instance, HspA1, HspB1, HspB5 (αβ-crystallin), Hsp90 and Hsp105 were also implicated to inhibit and regulate apoptosis. Indeed, the Hsps blocked both the intrinsic and the extrinsic apoptotic pathways through the interaction with main proteins at upstream of mitochondria, at the mitochondrial level and at the postmitochondrial level [38]. Moreover, the cytoprotective effects of Hsp70 and Hsp27 were related to their ability to hinder apoptosis [39]. Hsp70 could block apoptosis at the premitochondrial level by interaction with the death receptor-mediated signaling pathway (e.g., DR4 and DR5) inhibiting the TRAIL-induced assembly of the death-inducing signaling complex and BID activation; at the mitochondrial level by preventing mitochondrial membrane per-meabilization through the blockage of Bax translocation; and at the postmitochondrial level by suppressing the association between APAF-1 and procaspase 9 necessary to form the apoptosome, or by protecting nuclear proteins from caspase-3 cleavage. The reports showed that Hsp70 interacted with Mcl-1, a Bcl-2 family member, preventing mitochondrial events, as well [39,40]. In this line, a novel, small-molecule inhibitor of the cytosolic Hsp70 (VER-155008) could act similar to a small-molecule inhibitor of Hsp90, and potentiate its apoptotic potential in HCT116 colon carcinoma cells [41]. Moreover, Hsp72 modulated stress-activated signaling by directly inhibiting c-Jun N-terminal kinase (JNK) [42]. The results showed that the same concentration of Hsp72 was more efficient than Hsp27 at inhibiting cytochrome *c*-mediated caspase activation [43].

Hsp27 also blocked apoptosis at the premitochondrial level by inhibiting cytochrome *c* release indirectly through its action on F-actin, Bid or reactive oxygen species (ROS) and at the postmitochondrial level by directly binding to cytochrome c through the sequestration of cytosolic cytochrome *c*. Hsp27 also influenced apoptosis by favoring the ubiquitination/degradation of proteins IκBα (NF-κB inhibitor) or p27 under stressful conditions or by increasing the intracellular level of antioxidant glutathione [44]. The roles of Hsp27 in p53-mediated cellular responses to DNA damage are controversial. The phosphorylated Hsp27 (p-Hsp27) activated ATM-dependent p53 signaling and mediated the resistance of MCF-7 cells to doxorubicin-induced apoptosis [45]. On the other hand, downregulation of Hsp27 led to the activation of p53 and p21 likely due to the decreased level of unphosphorylated Hsp27 as a p53 suppressor. Indeed, the unphosphorylated Hsp27 modulated p53 nuclear importation process, and a negative correlation was found between Hsp27 and p53 [45]. The cell surface receptor Fas/APO-1 and its ligand were identified as important mediators of apoptosis. The results indicated that human Hsp27 was a cellular inhibitor of Fas/APO-1-induced apoptosis [46]. Also, the expression of Hsp27 inhibited the cellular death induced by TNF-α [47]. In contrast, another study represented that the overexpression of small Hsp B8 (HspB8) induced apoptosis through activation of TGF-β-activated kinase (TAK)-1, and p38 mitogen-activated protein-3 kinases in melanoma cell [48]. Therefore, the levels of Hsps and their functions are two major factors for induction or suppression of apoptosis.

As shown in Supplementary 1, the major stress proteins in mitochondria are Hsp60 and mtHsp70 that play an important role in inhibiting the aggregation of mitochondrial proteins. The mitochondrial Hsp60 stress protein was reported to play an important role in suppressing apoptosis by upregulation of the antiapoptotic molecules Bcl-2 and Bcl-xL as well as inhibition of caspase3 activation [49,50]. In general, overexpression of Hsp27, Hsp70, Hsp60 or Hsp90 suppressed apoptosis and prevented caspase activation in different cellular models following a variety of cellular stressors (Figure 1C [51]). Hsp90 could enhance the survival pathway regulated by Akt and BAD phosphorylation, inactivate the proapoptotic proteins Bax and Bak in the mitochondrial membrane and reduce the intrinsic apoptotic pathway by activation of procaspase-3. In this line, the researchers showed that Hsp90 inhibitors are important as therapeutic agents, especially anticancer drugs. Two leading small-molecule classes of Hsp90 inhibitors have progressed to clinical trials containing the ATP site-binding resorcinol moiety present in radicicol, and the purine scaffold series [52]. Some Hsp90 inhibitors used in clinical development include geldanamycin derivatives (e.g., tanespimycin (17-*N*-allylamino-17-demethoxygeldanamycin [17-AAG]), alvespimycin (17-DMAG), retaspimycin (IPI-504, IPI-493), resorcinol derivatives (e.g., ganetespib [STA-9090],

NVP-AUY922 [VER52296], AT-13387, KW-2478), purine analogs (e.g., BIIB021 [CNF 2024], MPC-3100, Debio 0932 [CUDC-305], PU-H71) and other synthetic inhibitors (e.g., SNX-5422, DS-2248, XL-888) [53]. Generally, Hsp90 inhibitors were actively pursued by the pharmaceutical industry with 17 agents having entered clinical trials [54]. The clinical activity of Hsp90 inhibitors suggested a potential cancer therapy against different oncogene-addicted cancer types that developed resistance to specific receptors [55]. Hsp90 inhibitors significantly showed antitumor activity in various preclinical tumor models. Although, promising clinical outcomes of these therapeutic agents were mainly observed in breast and lung cancer, however, their efficacy was relatively limited to date in clinical trials. Up to now, some significant toxicities and side effects have been reported [52]. For instance, PF-04929113 (SNX-5422) was a potent and highly selective small-molecule inhibitor of Hsp90 used in patients with advanced solid tumors and lymphomas, but its development was restricted due to ocular toxicity observed in animal models and in a Phase I human study [56]. In general, although none of Hsp90 inhibitors have received the US FDA approval, some of them have shown promising pharmacological and clinical activity [52].

HSPs in autophagy

Autophagy is an intracellular degradation system that delivers cytoplasmic components to the lysosome. This system protects cells from many types of stress and is thought to play a key role in preventing stress (Figure 1B [57]). Thus, autophagy is a catabolic process involved in the cell homeostasis [58]. For instance, mitochondrial autophagy protected the cells versus heat-induced apoptosis through a decrease in cytosolic cytochrome *c* release and caspase-3 activation [59]. Recent studies also indicated the crucial role of mitochondria and endoplasmic reticulum in the initiation of autophagy by using the autophagy proteins (e.g., ATG5 and light chain-3 [LC3]). In addition, mitochondria form tubular structures during serum starvation inducing autophagy. Alterations in autophagy-related genes were found in neurodegenerative and lysosomal storage disorders, and cancer types [37].

Generally, mitochondrial autophagy (mitophagy) is a selective form of autophagy, which is important in maintaining mitochondrial homeostasis. The results indicated that sHsps could protect NADH: ubiquinone oxidoreductase and NADH dehydrogenase activity during heat and oxidative stress in mitochondria [60–62]. In addition, the expression of HspB1 (/Hsp27) enhanced during oxidative stress in acute kidney injury. Indeed, overexpression of HspB1 reduced BAX activation and H_2O_2-induced apoptosis, and increased autophagic flux in renal tubular cells [57]. The studies showed that mitochondrial DNA mutations led to trigger some human neuromuscular diseases including an inherited encephalomyopathy so-called as Myoclonus epilepsy associated with ragged-red fibers (MERRF). According to the results, a significant decrease of Hsp27 was observed in lymphoblastoid and cytoplasmic hybrid (cybrid) cells of MERRF patients with A8344G mutation. The increased formation of microtubule-associated protein 1A/1B-LC3-II and autophagosomes was found in MERRF cybrids indicating a constitutively activated autophagic pathway. These data also showed that Hsp27 may be degraded by the autophagic pathway suggesting the fast turnover of Hsp27 and its protective role in MERRF cells. Thus, regulation of Hsp27 and autophagic pathway could be considered as an important solution to treat MERRF syndrome [63]. On the other hand, the researchers reported that the apoptosis can be accompanied with autophagy, by the increased expression of LC3 and the decreased value of lysosome pH. For example, nicotinic-mycoepoxydiene not only has an antitumor activity but also regulates cancer cell apoptosis and autophagy through Hsp90 inhibition [64]. Another study indicated that autophagy contributed to Hsp72-mediated cytoprotection in lipopolysaccharide (LPS)-associated peritonitis (peritoneal dysfunction) in a rat model. Exposure of the cultured peritoneal mesothelial cells to LPS resulted in autophagy followed by apoptosis. The data indicated that inhibition of autophagy reduced the antiapoptotic effect of Hsp72. Also, overexpression of Hsp72 enhanced autophagy through JNK phosphorylation and Beclin-1 upregulation, inhibited apoptosis and attenuated peritoneal injury. Indeed, suppression of JNK activity decreased Hsp72-mediated Beclin-1 upregulation and autophagy. These findings suggested that the induction of Hsp72 might be a potential therapy for peritonitis [65].

Innate & adaptive immune responses

The immunological functions of Hsps have been implicated in stimulation of innate (i.e., the NF-κB and interferon regulatory factor signaling pathways by Hsps alone), and adaptive (i.e., MHC-I and MHC-II pathways) immune systems (Figure 2A & B). The properties of Hsps in stimulation of immune responses were studied as following.

Innate immunity

Innate immunity is the first line of defense from infection in a nonspecific manner. Since 1993, Hsps such as Hsp60, Hsp70, Hsp90 and Gp96 purified from bacterial and mammalian sources as well as their recombinant products was shown to be potent inducers of the innate immune system through the secretion of proinflammatory cytokines including TNF-α, IL-1, IL-6 and IL-12, and the release of nitric oxide (NO) and C–C chemokines by antigen-presenting cells (APCs: macrophages and dendritic cells [DCs]). Moreover, Hsps-induced DC maturation by the upregulation of MHC class I and II molecules and costimulatory molecules (e.g., CD86 and CD80; Figure 2A) [66]. The studies demonstrated that the secretion of proinflammatory cytokines by Hsp60 and Hsp70 could promote chronic inflammation and autoimmune diseases [67]. On the other hand, the activation and maturation of DCs by Hsp70, Hsp90 and Gp96 not only stimulated the innate immune system but also activated effector natural killer (NK) cells [68]. Moreover, Hsps were considered as a 'danger signal' at the site of tissue injury, and could be the endogenous ligands for Toll-like receptors (e.g., TLR2 and TLR4) [69].

Adaptive immunity

Adaptive immunity plays a protective role against an infectious disease agent that is mediated by B- and T-lymphocytes following exposure to specific antigen, and characterized by immunological memory. For stimulation of adaptive immunity, Hsps bind to antigenic peptides and deliver them to the APCs for T-cell presentation through the MHC molecules. Indeed, immunization with HSP-peptide complexes could elicit potent antitumor, antiviral and antiparasitic effects along with clear immune mechanisms. The interaction of HSP–peptide complexes with APCs (i.e., macrophages or DCs) led to the presentation of antigenic peptides to $CD8^+$ and $CD4^+$ T lymphocytes (Figure 2B) [70]. The cross-presentation of peptides bound to HSPs was also shown to be receptor-mediated (e.g., CD91 for Hsp70/Gp96, scavenger receptor-A for Hsp90/Gp96, scavenger receptor-A, scavenger receptor-F1, stabilin-1, LOX-1 and SREC-1 for Hsp70 binding on APCs) inducing the secretion of different cytokines (e.g., IL-2, IFN-γ, etc.) [71]. Moreover, Hsp70 was expressed on the plasma membrane of tumor cells and/or tumor-derived exosomes. A report showed the possible adjuvant property of exosome Hsps that increased the maturation of DCs and NK cells resulting in immune system-mediated protection [72]. Some studies showed that Hsps could downregulate an immune response in autoimmune models eliciting IL-10-producing immunosuppressive T cells. For instance, epitopes derived from human Hsp40 stimulated differentiation and/or cell proliferation of human Tregs [73]. Furthermore, Hsp60 downregulated adaptive immune responses by stimulation of Tregs via TLR2 signaling leading to the inhibition of IFN-γ and TNF-α secretion, the upregulation of IL-10 in activated $CD4^+$ T cells, the increased differentiation of cord blood mononuclear cell into $CD4^+IL\text{-}10^+Foxp3^+$ Tregs, and T-cell immobilization through increased adhesion to fibronectin and decreased expression of chemokine receptors including CCR7 and CXCR4 [74]. On the other hand, Hsp70 could stimulate suppressive activity of Tregs through TLR4-signaling pathway resulting in Foxp3 induction and suppression of inflammatory reactions, and the secretion of anti-inflammatory IL-10 [75]. Interestingly, several studies indicated significant activation of antitumor cytotoxic T lymphocytes (CTLs) by the Hsp vaccines, followed by a delayed Treg response. These findings showed opposed effects of the carrier (i.e., Hsp) and cargo (i.e., antigenic peptide) as vaccine components on immunity. Indeed, the lower doses of chaperone vaccines could induce CTL responses more than the immunoregulatory response via decreasing the levels of Hsp-derived peptides below a threshold [76]. For further clarification, some various immunological studies of Hsps are described in detail in the following.

The potential use of Hsp90 inhibitors was studied in patients with autoimmune diseases where uncontrolled Th1 or Th17 activation occured. These inhibitors significantly decreased the proliferation of T cell, and secretion of proinflammatory IFN-γ, TNF-α and IL-17 cytokines. These effects were associated with inhibition of NF-κB p65 activity and upregulation of Hsp70 expression [77]. Other study also demonstrated that Hsp90 inhibitors irreversibly downregulated the co-stimulatory molecule (CD28, CD40L), and activating receptors on NK cells [78]. Geldanamycin, the first known Hsp90 inhibitor, modulated the proteasomal degradation of Hsp90 client proteins and displayed a potent antitumor activity *in vitro*, but it was very hepatotoxic for clinical use [79]. Geldanamycin downregulated CD25 on activated T lymphocytes, inhibited T-cell proliferation and decreased the cytotoxic activity of NK cells [80]. Moreover, the Hsp90 inhibitors could reduce total B cells in spleens, and induce high concentrations of IL-10 and low levels of circulating autoantibodies suggesting their use in clinical trials for treatment of autoantibody-mediated disorders [9]. Inhibition of Hsp90 blocked the activity of some viruses through different mechanisms. For instance, Hsp90 suppressed the replication of *Ebola* and *vaccinia* viruses through interaction with the core protein 4a [81,82], the cleavage of newly synthesized NSP2/3 protein in hepatitis C virus (HCV), the activity

of reverse transcriptase in HBV [83,84], and proper folding of the capsid protein in *polio virus* [85]. Generally, the studies indicated that Hsp70 and Hsp90 differentially regulated translocation of extracellular antigen to the cytosol for cross-presentation [86]. Indeed, the Hsp70 inhibitors (e.g., VER) blocked both endogenous antigen presentation and cross-presentation. In contrast, the Hsp90 inhibitors (e.g., radicicol) suppressed cross-presentation completely (i.e., translocation of antigen from endosome to cytosol), but showed only partial inhibition of endogenous antigen presentation [87,88].

Hsp60 was shown to stimulate production of proinflammatory cytokines and other proteins involved in inflammation, as well. For instance, the mycobacterial Hsp60 (~65 kDa) activated monocytes to secrete proinflammatory cytokines (e.g., IL-6, IL-8 and TNF-α) and NO, and induced IL-12 and IL-15 production, antigen-specific IFN-γ secretion and CD69 expression on $CD4^+$ T cells by APCs [89]. On the other hand, Hsp60 enhanced the function of $CD4^+CD25^+$ Treg through innate TLR2 signaling leading to activation of protein kinase C, phosphoinositol-3-kinase, p38 MAP kinase and secretion of TGF-β and IL-10 [90]. Thus, Hsp60 could downregulate adaptive immune responses by upregulating Tregs. A study indicated that the expression and secretion of Hsp60 was activated by PDGF-BB and IL-8 in both human umbilical vein endothelial cells and vascular smooth muscle cells via TLR4 and ERK MAPK activation [91].

Asea *et al.* showed that treatment of human DCs with Hsp70 and LPS augmented the proliferation of immature DCs compared with mature DCs, the secretion of proinflammatory cytokines (e.g., IL-12, IL-1, TNF-α), and the expression of costimulatory molecule on DCs. These results indicated an important role of Hsp70 in linking innate and adaptive immune responses [92]. The studies demonstrated that the immunogenic Hsps such as Gp96, Hsp70 and calreticulin could bind to CD91 on APCs for cross-presentation of the Hsp-chaperoned peptides leading to the maturation of APCs, secretion of cytokines and priming of T-helper (Th) cells, and activation of NF-κB in a dose-dependent manner [93]. Hsp70 was found to activate macrophages and other cells via TLR4 or TLR2 signaling [94]. Indeed, the extracellular Hsp70 and Hsp60 could enhance macrophage-mediated antigen uptake and induce the production of proinflammatory cytokines including TNF-α, IL-1 and IL-6 in monocyte and macrophage [95]. Moreover, Hsp70 and Hsp65 generated β-chemokines that function as innate adjuvants enhancing adaptive immunity [96]. Giraldo *et al.* showed that exercise-induced extracellular Hsp72 could stimulate neutrophil phagocytic and fungicidal potency through TLR-2 pathway [97]. In addition, Hsp70-like protein 1 (hsp70L1), a novel HSP derived from human DCs, was able to activate DCs and act as a Th1-polarizing adjuvant. Hsp70L1 (~54.8 kDa) is smaller than Hsp70 but resembles it both structurally and functionally. Hsp70L1 could induce DC maturation and stimulate secretion of the proinflammatory cytokines such as IL-12p70, IL-1, TNF-α and the chemokines IP-10, MIP-1 and RANTES. Hsp70L1 could stimulate IL-12p70, CC-chemokine and CCR7 and CXCR4 expression more efficiently than Hsp70 [98]. Interestingly, a study indicated that Hsp40 and Hsp70 equally regulate HIV-1 gene expression and replication. As observed, the level of HspBP1 was significantly downregulated during HIV-1 infection. Indeed, HspBP1 could act as an endogenous negative regulator of HIV-1 gene expression and replication by suppressing NF-κB-mediated activation of viral transcription [99]. Other study showed that HspA8 (so-called as Hsc70, Hsc71, Hsp71 or Hsp73) was overexpressed at the cell surface of tumors. This protein was recognized by T cells carrying γδ T-cell receptor. Indeed, HspA8 may present peptides to $CD3^+$ $CD4^-$ $CD8^-$ T-cell receptor $\alpha\beta^-$ NK T cells [100]. On the other hand, unphosphorylated HspB1 (Hsp27) formed a complex with kinase AKT and MAPKAP kinase-2 in neutrophils that could prevent neutrophil apoptosis and induce an inflammatory response. HspB1 stimulated the secretion of IL-10 in monocytes, and thus could suppress the immune response. Indeed, the extracellular HspB1 inhibited the differentiation of monocytes toward macrophages and DCs and also their maturation [101]. In addition, Hsp27 regulated the release of proinflammatory mediator in keratinocytes by modulating NF-κB signaling. Downregulation of Hsp27 increased prostaglandin E2 production, and the release of the proinflammatory cytokines COX-2, IL-6 and IL-8 in keratinocytes [102]. Treatment of macrophages (e.g., the THP-1 human monocytic cell line) with the recombinant HspB1 (rHspB1) resulted in the expression of the proinflammatory factors such as IL-1β and TNF-α, and also the expression of the anti-inflammatory factors such as IL-10 and GM-CSF both at the mRNA and protein levels [103].

HSPs & cell surface receptors in immune system

The biological effects of extracellular Hsps are mediated through their interactions with the cell surface receptors such as CD91 known as α2-macroglobulin receptor, low-density lipoprotein (LDL) receptor-related protein-1 and apolipoprotein E receptor. CD91 was often expressed in hepatocytes and vascular smooth muscle cells [104]. At first, CD91 was identified as the first endocytic receptor for the cross-presentation of Gp96–peptide complexes. The

next studies showed a major role for CD91 in the cross-presentation of peptides associated with a variety of Hsps such as Hsp90, Hsp70 and calreticulin [105]. Furthermore, Hsp70 could interact with lectin-type oxidized LDL receptor (LOX-1) on the surfaces of human DC leading to the cross-presentation of associated tumor antigens and stimulation of $CD8^+$ T lymphocytes [106]. Moreover, LOX-1 clusters with the SR, a group of proteins that bind to oxidized LDL and acetylated LDL. A significant interaction of Hsp70 with at least two other members of the SR family, including SREC-I and Stabilin-1/FEEL-1 was observed [107,108]. Hsp90–peptide complexes could also interact with murine bone marrow-derived DCs using both LOX-1 and SREC-I receptors. SREC-I mediated the uptake of peptides into the MHC class II pathway and subsequently induced the activation of $CD4^+$ T cells [109,110]. In addition, interaction of SREC-I with TLR4 was observed on the surface of mouse macrophages [108]. On the other hand, TLRs, especially TLR2 and TLR4, mediated inflammatory responses to Hsps [111]. A study indicated that sialic acid-binding immunoglobulin-like lectins (Siglecs) as a receptor family may result in inflammatory responses after binding to Hsps [112]. Upon activation, Siglec receptors could directly associate with TLRs and inhibit TLR-mediated activation of inflammatory signaling cascades such as the NF-κB pathway [113]. In addition, CD40 was reported as a signaling receptor for mycobacterial but not mammalian Hsp70, and also as an endocytosis receptor for murine Hsp70. Human CD36 was also shown to confer the ability to bind Gp96 [114].

Treatment

Hsps showed important implications in general health and were proposed to be therapeutic targets. Supplementary 2 shows major applications of Hsps in vaccine development and diagnosis. None of the described results changed the common standard treatment in any of the cited diseases within the clinic. Hsps have different roles in anticancer effects, drug resistance and vaccine design as described in below.

Anticancer effects of HSPs

Members of the small HSP family (e.g., Hsp27 and αβ-crystallin) indicated cardio- and neuro-protection, potent antiapoptotic activity, proangiogenic, anticancer and anti-inflammatory properties involving interactions with several clients [115]. In addition, Hsp90 was emerged as a potential therapeutic target for cancer. A study indicated that Hsp90B1 (known as endoplasmin, Gp96, Grp94), a member of HSP90 family, was a direct target of miR-223 that was abnormally upregulated in some cancers including ovarian, bladder and colorectal cancers. In contrast, miR-223 was downregulated in osteosaroma. The miR-223 could play a tumor-suppressor role in osteosarcoma through the phosphoinositol-3-kinase/Akt/mTOR pathway. Indeed, the tumor cells showed significant G0/G1 arrest and increased apoptosis due to gene silencing [116].

Dual targeting of Hsp90 and Hsp70 induced cell death and enhanced the anticancer effect of chemotherapeutic agents in advanced bladder cancer. For example, the Hsp90 inhibitor, 17-AAG, increased the antiproliferative and apoptotic effects of chemotherapeutic agents, suppressed Akt activity and stimulated the upregulation of Hsp70. Also, the Hsp70 inhibitor, pifithrin-μ, enhanced the effect of 17-AAG and chemotherapeutic agents [117]. Other study indicated that Hsp27 was significantly upregulated in angiogenic cells as compared with nonangiogenic cells. Indeed, downregulation of Hsp27 reduced the proliferation of endothelial cell, and the secretion of VEGF-A, VEGF-C and basic FGF. In contrast, overexpression of Hsp27 in nonangiogenic cells resulted in tumor growth *in vivo*. Thus, targeting Hsp27 could be considered as an important strategy in cancer treatment by determining its levels for the balance between tumor dormancy and tumor progression [118]. The Hsps 27, 40 and 70 were used as combinational and dual therapeutic cancer targets and could decrease the possibility of chemotherapeutic drug resistance as compared with the used monotherapies [119]. On the other hand, the stress-induced Hsps could also act as endogenous danger signals. For example, the Hsps (Hsp60, Hsp70, Hsp90)-harboring exosomes secreted by anticancer drug-treated human hepatocellular carcinoma cells (e.g., HepG2 cells) effectively induced Hsp-specific NK-cell cytotoxic responses and Granzyme B secretion, upregulated the expression of CD94 inhibitory receptor, and downregulated the expression of CD69, NKG2D and NKp44 activating receptors. This strategy can be considered as a potent strategy for vaccine development against hepatocellular carcinoma [120].

Drug resistance

The reports showed that Hsps were induced by several cytotoxic drugs *in vitro*. These proteins were involved in anticancer drug resistance [121]. For instance, high levels of Hsp90/ p23 cochaperone could induce tumor progression in breast cancer by increasing lymph node metastases and drug resistance [122]. Moreover, the correlation of Hsp27, Hsp70 and Hsc70 expression with drug resistance was evaluated in breast cancer patients treated with chemotherapy.

Indeed, nuclear expression of Hsp27 and Hsp70 was increased and cytoplasmic expression of Hsp70 and Hsc70 was decreased after chemotherapy. The results indicated that drug resistance was significantly correlated with a high nuclear level of Hsp70 in tumor cells. Moreover, the combination of Hsp27 and Hsp70 levels showed a strong correlation with disease-free survival in patients. Thus, these data indicated that Hsp27 and Hsp70 were involved in drug resistance in breast cancer patients treated with combination chemotherapies [121]. On the other hand, the relationship between Hsp70 and human EGF receptor-2 (ERBB2) expression in breast cancer cell lines was associated with drug resistance [123]. Moreover, the evaluation of Hsp27 level in the intrinsic resistance of human colon cancer cells to doxorubicin indicated a good correlation between cell survival after doxorubicin treatment and Hsp27 content. However, an increased level of Hsp27 after cisplatin treatment was not associated with a decreased cytotoxicity to doxorubicin [124]. The overexpression of Hsp27 under stress conditions including heat shock and drug treatment (e.g., cisplatin or doxorubicin) in a human testis tumor cell line (833K) also showed that these cells were more resistant to heat and drugs related to a small decrease in the number of S-phase cells. Thus, the low constitutive levels of Hsp27 could increase the cellular sensitivity to chemotherapy in testis tumor cells [125]. A study indicated that cisplatin-induced expression of small Hsp25 in Ehrlich ascites tumor cells resulted in thermotolerance [126]. In contrast, the thermotolerant cells raising the levels of Hsp27 and other Hsps did not represent an increase in resistance to cisplatin *in vitro* [127] or *in vivo* [128]. It was shown that upregulation of Hsp27 induced resistance to 17-AAG, an anticancer agent in Phase I and II clinical trials through a glutathione-mediated mechanism [129].

Hsp-based vaccines

Hsp-based vaccines are emerging as a novel therapeutic approach in cancer and other diseases. HSPs possess significant properties to target DCs including: they are natural adjuvants; they deliver multiple antigens that can induce adaptive immune responses and they are safe components of existing vaccines [130]. Due to Hsp chaperone activity, Hsp-bound tumoral peptides could be released in the extracellular medium under stress conditions or immunological danger signals. HSPs could interact with the APC through different receptors (i.e., CD91, CD40 and LOX-1). After endocytosis, the Hsp-peptide complexes were degraded and tumoral peptide could be cross-presented to $CD8^+$ T cells through MHC I molecules [131]. The studies indicated that determination of optimal doses of Hsps is critical to administer to patients. Vitespen is an autologous cancer vaccine derived from tumor-specific Gp96 that showed promising results in Phase III clinical trials in melanoma and kidney cancer [132,133]. However, some experiments indicated that the cytokine effects of Hsps may be due to the contaminating LPS and LPS-associated molecules in Hsp preparation (e.g., the recombinant Hsps generated in bacterial expression system and/or bacterial Hsps). This subject is an important point before using Hsps as therapeutic agents [134]. In addition, immune responses induced by Hsps purified from tumors depend on the maintenance of Hsp-bound peptides. Thus, determination of peptide dose associated with Hsps is critical. This subject is problematic to use Hsp-peptide complexes in clinical trials. For example, oncophage (vitespen or HSPPC-96) was prepared from the patient's own tumor containing Hsp–peptide complex as an adjuvant treatment for patients with localized renal cell carcinoma. Herein, the immune response to vitespen was tumor/patient specific and was dependent on the peptide component of vitespen. These problems were major reasons for the rejection of vitespen approval (www.ema.europa.eu/docs/en_GB/document_library/Application_withdrawal_assessment report/2010/03/WC500075459.pdf).

Recently, Hsp molecules were utilized in DNA- or protein (peptide)-based vaccines, as antigens or adjuvants, against cancer and infectious diseases [12]. Several Hsp-based vaccines are underway in preclinical and clinical studies. For example, a tuberculosis vaccine containing Hsp complex from BCG (T-BioVax) showed high efficacy in the mouse *Mycobacterium tuberculosis* aerosol challenge model [135]. Moreover, a vaccine containing 32 synthetic HSV-2 peptides noncovalently complexed with the recombinant human Hsp70 protein (HerpV) was safe and well tolerated in human. HerpV was the first Hsp-based vaccine to elicit significantly $CD4^+$ and $CD8^+$ T-cell immune responses against HSV-2 antigens [136]. Supplementary 2 shows the use of Hsps in preclinical and clinical trials. Generally, the immunogenicity of Hsps results from two different properties: peptide-dependent ability to chaperone and elicit adaptive CTL responses against antigenic peptides, and peptide-independent immunomodulatory potency. The studies showed that certain Hsps including Hsp70 and Gp96 are highly effective carrier molecules for cross-presentation [8].

Hsps as antigen for the stimulation of immune responses

Hsps function as an antigen for immune stimulation. For example, the intratumoral injection of Hsp70 and/or upregulation of Hsp70 within the tumor showed a promising therapeutic approach in preclinical trials [137]. It was observed that an intratumoral vaccination with the recombinant oncolytic type-2 adenovirus overexpressing Hsp70 could suppress the growth of tumors via Hsp70-mediated immune responses and high oncolytic activity [138]. Hsp peptide-specific CTLs effectively reduced tumor burden in the xenograft mouse model of myeloma. Indeed, DCs pulsed with Hsp27 and Hsp90 peptides were used to stimulate peripheral blood mononuclear cells from myeloma patients, and subsequently to generate Hsp peptide-specific CTLs [139]. A chaperone-based vaccine was produced by purification of Hsp70-peptide complexes from fusion of DCs and tumor (Hsp70.PC-F). This vaccine could significantly induce immune responses including DC maturation and CTL activity against tumor cells in animal model [140]. In a study, Hsp70-peptide complexes were purified from human melanoma cell lines A375, A875, M21, M14, WM-35 and SK-HEL-1 (named as M-Hsp70-PCs). The data indicated that mature DCs pulsed with M-Hsp70-PCs induced the secretion of IFN-γ and CTL responses compared with the autologous Hsp70-PCs in patients [141,142]. The studies showed that a novel small HSP (~20 kDa) conserved in the hemoprotozoan parasite *Babesia botis* and *B. bigemina* stimulated memory $CD4_+$ T-lymphocyte responses and IFN-γ production in *B. botis*-immune cattle [143]. Moreover, the recombinant Hsp20 of *B. orientalis* (rBoHsp20) was an immunodominant antigen as a potential vaccine candidate, and could be a useful diagnostic reagent to detect antibodies against *B. orientalis* in water buffalo [144]. On the other hand, 62% of the *Leishmania*-infected animals developed significant humoral responses against the Hsp20 designed as DNA vaccine. In contrast, few sera from leishmaniasis patients showed a positive reactivity against the recombinant Hsp20 (rHsp20), suggesting that this protein is poorly antigenic for human immune system [145]. Also, Hsp20 was intracellularly expressed in all merozoites. In a study, the truncated rHsp20 proteins and overlapping peptides were tested to stimulate T cells from immune cattle. Both the N-terminal (aa 1-105) and C-terminal (aa 48-177) regions of Hsp20 from merozoites were immunogenic for the majority of cattle stimulating strong proliferation and IFN-γ production [146]. Another study described the development of a recombinant strain of modified vaccinia Ankara expressing a chimeric antigen containing B and T-cell epitopes from three antigenic proteins of *B. bovis* including MSA-2c, RAP-1 and Hsp20 that was evaluated as a candidate vaccine in homologous and heterologous prime-boost immunizations. The best vaccination strategy was achieved with a prime of protein cocktail and a boost with the recombinant virus inducing high levels of specific IgG antibodies and IFN-γ, and a high degree of activation of $IFN\text{-}\gamma^+$ $CD4^+$ and $CD8^+$-specific T cells for protection against bovine babesiosis [147]. The researchers also demonstrated the role of bacterial Hsps in vaccine design against various infectious diseases. For instance, vaccination with Hsp complexes, in other words, the GroEL/S (58 kDa also called HspB/Hsp60 and 13 kDa also called HspA, respectively) and the Dna K/DnaJ (also called Hsp70 and Hsp40, respectively) induced protection against *Helicobacter pylori* without exogenous adjuvant in mice. The Hsp complex (HspC) vaccines containing Hsp derived from pathogenic bacteria induced immune responses against their chaperoned proteins without addition of an exogenous adjuvant [148]. For instance, vaccination with *H. pylori* HspC could elicit high levels of antibodies and cytokines, and significantly cause protection against the next challenge with this pathogen without the induction of a severe inflammatory response [148]. On the other hand, Hsp70 from *Trichinella spiralis* (Ts) induced protective immunity in BALB/c mice by activating DCs. The results demonstrated that the recombinant Ts-Hsp70 activated DC maturation characterized by the secretion of IL-1β, IL-12p70, TNF-α and IL-6, and the increased surface expression of MHC-II, CD40, CD80 and CD86. The rTs-Hsp70-activated DCs induced the secretion of both Th1 (IFN-γ and IL-2) and Th2 (IL- 4 and IL-6) cytokines in $CD4^+$ T cells from *T. spiralis*-infected mice. The group vaccinated with rTs-Hsp70-activated DCs showed a 38.4% reduction in muscle larvae compared with the group vaccinated with DCs, alone [149]. Moreover, immunization of BALB/c mice with a *Mycobacterium leprae* Hsp65-based DNA vaccine (GroEL2) elicited both IFN-γ production and potent antibody responses and protective efficacy against *Buruli ulcer* [150].

Hsps as an adjuvant for the stimulation of immune system

Adjuvants can stimulate innate immune responses, and modify the quantity and quality of adaptive immune responses depending on the activated type of innate responses [151]. Hsps have been used as potent adjuvants for stimulation of immune system (e.g., CTL response) against cancer and infectious diseases. Some studies showed that immune activities reside within N- or C-terminal fragments of Hsps. Thus, these small fragments or minichaperones can be used in cancer immunotherapy and vaccine development [152]. Different studies have indicated that Gp96 and its N-terminal fragment (NT-gp96) could serve as potential adjuvants to enhance the peptide-specific CTL

responses in mice (e.g., against HBV infection and hepatocellular carcinoma [HCC]) [152,153]. Li *et al.* showed that NT-gp96 significantly enhanced humoral immune response induced by HBsAg [154]. Chen *et al.* also confirmed that NT-gp96 was an effective immunoadjuvant in antigen-specific humoral immune responses elicited by B-cell epitopes of porcine reproductive and respiratory syndrome virus in swine [155]. Moreover, the adjuvant activity of the N-terminal domain of Gp96 was more efficient in the induction of immune responses when fused to the C-terminal end of a polytope (PT) HCV DNA vaccine. The data showed that immunization of CB6F1 mice with PT DNA vaccine fused to NT-gp96 significantly induced the IFN-γ and TNF-α secretion, and antibody responses (IgG2a followed by IgG1) compared with PT DNA alone [156]. Moreover, NT-gp96 could enhance immunity potency of the recombinant HCV NS3 protein through production of proinflammatory cytokines [157]. Other results showed that the codelivery of human papillomavirus (HPV) E7 with the full length of Gp96 as DNA/DNA and of E7 with the C-terminal fragment of Gp96 (CT-gp96) as DNA/protein could stimulate E7-specific immune responses in C57BL/6 mice [158]. Gong *et al.* used human liver Gp96 and its N-terminal fragment (N336, amino acids 22-336) as an adjuvant for p24 protein or its HLA-A2-restricted peptide (FLQSRPEPTA) in mice. In fact, mice immunization with the recombinant p24-N336 fusion protein induced p24-specific antibody responses. Also, immunization of HLA-A2 transgenic mice with FLQSRPEPTA-gp96 mixture stimulated peptide-specific CTL responses [159]. Another study indicated the protective efficacy of the recombinant *Leishmania tarentolae* expressing KMP11-NT (gp96)-GFP fusion as a live-engineered recombinant vaccine candidate against visceral leishmaniasis in BALB/c mice [160]. Our previous results also showed that subcutaneous administration of mice with the recombinant *L. tarentolae* expressing E7-CT (gp96) fusion (*L. tar*-E7-CT-gp96) led to increase the levels of IFN-γ and also IgG2a before and after challenge with TC-1 tumor cells and also significant protective effects as compared with group vaccinated with *L.tar*-E7 [161]. Furthermore, our previous data demonstrated that subcutaneous injection of E7 DNA fused to CT (gp96) fragment followed by electroporation could notably generate effective immune responses and increase the efficiency of DNA vaccines against tumors [162]. Another study showed that fused adjuvant-free E7-NT-gp96 protein vaccination could direct the immune responses toward Th1 immunity and enhance protective antitumor immunity as compared with E7 protein alone [163].

Zhang *et al.* evaluated antitumor responses against fusion proteins of Hsp70 with tumor-associated antigen. They showed that the C-terminal peptide-binding domain of Hsp70 was essential in inducing antitumor response and NK cell activation against challenge with murine tumor B16 expressing Mela tumor antigen [164]. In contrast, a study showed that fusion of HPV E7 to the N-terminal fragment of the mouse Hsp70 induced an E7-specific CTL response and protected mice against tumor challenge. Herein, $CD4^+$ T cells and NK cells did not have any important role. This study indicated that the peptide-binding region of Hsp70 was required for the potency of E7-Hsp70 DNA vaccine. Generally, functional domain of Hsp70 and orientation of the E7 linkage to Hsp70 have clinical importance for the optimization of Hsp70-based DNA vaccines [165]. In chronic nonprogressive pneumonia, elongation factor *Tu* and Hsp70 are membrane-associated proteins from *Mycoplasma ovipneumoniae* eliciting increased levels of IgG, IFN-γ, TNF-α, IL-12, IL-4, IL-5 and IL-6 in BALB/c mice. Indeed, the recombinant Hsp70 could act as a Th1 cytokine-like adjuvant in mice [166]. Han *et al.* showed that the complexes of trophoblastic peptides and Hsp70 (as a novel contraceptive vaccine) activated T cells directed toward Th1 and specifically caused cytolysis of trophoblasts leading to the termination of pregnancy in a mouse model [167]. Similarly, the endotoxin-minimized HIV-1 p24 fused to murine Hsp70 induced p24-specific Th1 response (i.e., high IFN-γ and IgG2b) in mice [168].

Small Hsp27 was also proposed as an effective adjuvant for enhancement of HIV-1 Nef antigen-specific immunity. The Hsp27-Nef fusion protein significantly increased the Nef-specific T-cell responses inducing high levels of IgG2a and IFN-γ directed toward Th1 responses and also Granzyme B secretion. The immunostimulatory properties of Hsp27 were significantly more than Freund's adjuvant in different immunization strategies suggesting the use of Hsp27 for improvement of HIV-1 Nef-specific B- and T-cell immune responses in protein-based vaccines [169]. Moreover, Hsp27 could be used as a suitable carrier in DNA vaccine design against HIV-1 infections [170].

Some studies utilized bacteria-derived Hsps in vaccine development. For example, administration of a tuberculosis DNA vaccine encoding Hsp65 of *Mycobacterium tuberculosis* and the human IL-2 fusion (HSP65-IL-2 DNA vaccine) could enhance Th1-type cellular responses by producing greater levels of IFN-γ and IL-2 with a higher titer of antigen-specific anti-Hsp65 IgG2a as well as high antigen-specific cytotoxicity activity [171]. In addition, immunization of mice with the E7 antigen fused to Hsp65 markedly changed the E7 recall response from IL-5 to IFN-γ production suggesting a shift toward a type-1 response [172]. Another study showed that the *M. tuberculosis* Hsp X (HspX) could significantly increase the expression of CD86 and MHC class II, and the secretion of proinflammatory cytokines in DCs through the MyD88- and TRIF-dependent pathways. Indeed, systemic

administration of HspX-E7-DCs induced a significant suppression of tumor growth in mouse model. Thus, HspX can be considered as an effective immunoadjuvant in DC-based tumor immunotherapy [173,174].

Diagnosis

Hsps as a biomarker in noninfectious & infectious diseases

Several studies showed the increased levels of Hsps in patients suffering from infectious diseases suggesting their role as promising biomarkers [175]. Cancer biomarkers are usually classified into three categories: prognostic, predictive and pharmacodynamic. Tumor biomarkers in the blood are useful to monitor tumor growth and to assess the effects of anticancer therapies by noninvasive methods [176]. Among Hsps, Hsp70 is often overexpressed in tumor cells and can be actively released in exosome-like lipid vesicles. The assessment of liposomal and free Hsp70 in serum and plasma using ELISA could provide a useful tool for detecting tumors and monitoring the clinical outcome of patients [177]. Moreover, the soluble Hsp70 was helpful to detect tumors at early stages. Validation of Hsp70 as a tumor-specific biomarker was studied for monitoring the outcome of radiation therapy in tumor mouse models and in cancer patients [178–180]. Abe *et al.* indicated that Hsp70 is a marker of prostate cancer. Although, the levels of plasma Hsp70 were not more effective than prostate-specific antigen (PSA) as a predictor for diagnosis of patients with prostate cancer, but its use along with PSA might be useful in identifying patients with early-stage prostate cancer who might be missed by PSA screening, alone [181]. Other results suggested that four members of the HSP70 family, in other words, two glucose-regulated proteins (Grp78 and Grp75), heat shock cognate protein (Hsc70) and Hsp70 protein 1 (Hsp70.1) play important roles in the pathogenesis of HCV-related HCC, and could be molecular targets for diagnosis and treatment of disease [182]. Hsp27 as a potential biomarker could help in the diagnosis of HCC [183]. Detection of novel tumor-related antigens and autoantibodies in cancer patients could facilitate the diagnosis of early-stage malignant tumor and determine the efficacy of novel immunotherapies. In this line, an autoantibody against Hsp70 in sera from patients with esophageal squamous cell carcinoma offered a strong tool for identifying novel serum markers that may display clinical use against cancer [184]. Hsp70 was frequently overexpressed by bladder cancer cells and could be used as biochemical marker in patients with bladder cancer [185]. The investigators found that the increased levels of Hsp60, Hsp70 and Hsp90 could distinguish the patients with muscle-invasive bladder cancer (MIBC) from those with nonmuscle-invasive bladder cancer (NMIBC). However, high levels of the Hsps could not distinguish patients with NMIBC from those with hematuria and without bladder cancer. In contrast, the evaluation of Hsp60 and IL-13 levels simultaneously could detect all cases of MIBC, and most cases of NMIBC [186]. Pick *et al.* indicated that the expression of Hsp90 in malignant cells was increased about two- to tenfold higher than in normal cells indicating its important role in the growth and survival of tumor cells which could be considered as an effective drug target [187].

The expression of sHsps was modulated in diseases such as Alzheimer's, Parkinson's and cancer. Circulating αB-crystallin and Hsp27 in the plasma could exhibit immunomodulatory and anti-inflammatory functions [115]. Hsp27 was critical for dynamic intracellular trafficking during autophagy and mitophagy as a cytoskeleton regulator [60,62]. The p-Hsp27 was used as a potential biomarker to predict the role of chemotherapy-induced autophagy in osteosarcoma response to therapy. The increased p-Hsp27 was associated with high sensitivity to anticancer drugs when autophagy was inhibited. The results revealed that the p-Hsp27 could represent a predictive biomarker of whether combination therapy with autophagy modulators and chemotherapeutic drugs will be useful for overall survival of patients [58]. A study indicated that the mean serum levels of small Hsp27 and Hsp60 in cancer patients and patients with myocardial infarction was significantly higher than healthy individuals [188]. Plasma concentrations of Hsp70 increased with the progression of heart failure and could act as a potential screening biomarker for early diagnosis of heart failure [189]. On the other hand, the concentrations of anti-Hsp20 antibody were inversely correlated with tumor progression in ovarian cancer [190]. The current studies demonstrated abnormal expression of Hsps in a variety of human tumors such as ovarian, colon, breast, endometrial, lung and prostate tumors. In contrast, the expression of Hsps showed a poor prognosis of patients with colorectal and gastric cancers [191]. Rappa *et al.* showed that Hsp10 and Hsp60 are promising biomarkers for early diagnosis of tubular adenoma in both epithelium and lamina propria, and its differentiation from more advanced malignant lesions. Thus, Hsp10 and Hsp60 could be potentially considered as convenient targets for therapy [192].

The identification of serological markers could also facilitate the diagnosis of viral infections. The results indicated that patients with high antibody response to HPV16 E7 had significant seroreactivity to CT-gp96 fragment. Indeed, the evaluation of cervical cancer patients' seroreactivities against three recombinant proteins (i.e., rE7, rNT-gp96 and rCT-gp96) showed significantly higher levels of these markers in squamous cell carcinomas only, but not

in adenocarcinoma and control groups [193]. A recent study indicated the correlation of HBcAg-specific IL-10-secreting Treg cells and the serum levels of Hsp60 in patients with chronic HBV. As observed, the serum level of Hsp60 in patients with chronic HBV was significantly higher than that in patients with chronic HCV. Moreover, preincubation of $CD4^+$ $CD25^+$ cells with the recombinant Hsp60 significantly enhanced the frequency of HBcAg-specific IL10-secreting Tregs [194]. The data indicated that host Hsp70 and Hsp90 have potential in the diagnosis of tuberculosis meningitis disease with good sensitivity and specificity. The combined use of all Hsps (Hsp25, Hsp60, Hsp70 and Hsp90) could effectively distinguish patients with tuberculosis meningitis from controls [195].

Correlation between Hsp mutation & disease

Mutations in human sHsps resulted in myopathies, neuropathies and cataract. For example, mutation-decreasing chaperone activity in αB-crystallin led to desmin-related myopathy and congenital cataract [196]. Chaperonopathies were divided into acquired or genetic diseases. They were caused by aging or post-translational modifications. For instance, the expression of αA-crystallin was decreased with age, and its post-translational changes in the retina led to an acquired chaperonopathy known as retinopathy [197]. Mutations in five members of the HSPB family (i.e., HspB1, HspB3, HspB4, HspB5 and HspB8) developed muscular and neurological disorders. Most of these mutations were located in the highly conserved α-crystallin domain responsible for stabilization of the homo- or hetero-oligomers. Indeed, mutations in HspB1 (14 mutations), HspB3 (one missense mutation) and HspB8 (two mutations) were associated with hereditary peripheral neuropathies [198]. Also, mutations in HspB4 (10 mutations) and HspB5 (13 mutations) were associated with congenital cataract [198–200]. In general, the mutations found in the HspB1 and HspB3 were associated with development of certain neurodegenerative diseases. The mutations of HspB4 were involved in development of cataract. Multiple effects of HspB5 mutations were also accompanied by development of different congenital diseases such as cataract and different types of myopathies. The HspB6 and HspB8 mutations could affect the overall structure of proteins [201].

A mitochondrial chaperone known as HspA9, HspA9B, mortalin and mortalin 2 (MOT/MOT2) or GRP75 was necessary for the proteolytic activity of LONP1, a mitochondrial chaperone and protease, and an agent of the human CODAS (cerebral, ocular, dental, auricular and skeletal) syndrome. The biallelic mutations found in *HspA9* gene encoding mHsp70/mortalin were associated with a family of mitochondrial chaperonopathies and human embryonic morphogenesis [202]. Adriaenssens *et al.* showed that the mutations in the coding regions of HspB1 (Hsp27) and HspB8 (Hsp22) cause distal hereditary motor neuropathy and Charcot-Marie-Tooth (CMT) disease. Recently, the clinical spectrum of HspB1 and HspB8 mutations were accompanied by development of myopathies and neuromuscular diseases [203]. Mutations in the *HspB3* gene were reported as a putative cause of distal hereditary motor neuropathy 2C and CMT type 2 (CMT2) family. Also, mutations in the *HspB1* and *HspB8* genes were reported to be associated with both types of CMT2 and distal hereditary motor neuropathy [204].

Conclusion

Generally, different studies showed that Hsps have several important roles in both prokaryotic and eukaryotic systems. Hsps plays the key roles in apoptosis, autophagy, protein folding and turnover, drug resistance, diagnosis and treatment. Moreover, Hsps are used as an antigen and/or adjuvant for stimulation of immune responses in vaccine development. Hsps induce both innate and adaptive immunity through interaction with various receptors on APCs. On the other hand, Hsps are used as a biomarker in diagnosis of noninfectious and infectious diseases. Some Hsp mutations are correlated with a variety of diseases. Indeed, Hsps exhibit cardio- and neuro-protection, and potent anticancer properties. The reports indicated that the levels of Hsps are critical for immunological and biochemical activities such as immune stimulation, apoptosis, autophagy and drug resistance.

Future perspective

Hsps play a critical role in the maintenance of cellular homeostasis through interaction with different signal pathways. Hsps protect the cell components against stress via prevention of the protein aggregation, maintenance of the membrane stability, inhibition of the apoptotic pathway and reduction of oxidative stress. Some Hsps need to interact with specific cochaperones as a part of large protein machinery. High expression of Hsps was reported in variety of cancers including breast, prostate, colorectal, lung, ovarian, gastric, oral and esophageal cancer. Indeed, detection of their specific antibodies in the sera of patients could play an important role in cancer diagnosis. Thus, Hsps could be used as a clinical biomarker and therapeutic target in several infectious and noninfectious diseases. HSPs were determined as a novel anticancer therapy. The studies showed that the combination of different

HSP inhibitors may overcome the acquired resistance observed in Hsp monotherapy and subsequently generate synergistic cytotoxic effects. On the other hand, the immunogenicity of HSPs suggests them as a promising candidate antigen for vaccine development. Many HSP-based cancer vaccines showed potential and hopeful results in both preclinical and clinical trials. However, further studies are required to develop novel and effective Hsp-targeting strategies for treatment and diagnosis of different disorders as well as identification of Hsp mutations associated with human-inherited disorders. Regarding to their effective immune stimulatory function, regulating both innate and adaptive immune signals, Hsps can be considered as major components of immunotherapy in the future.

Executive summary

Heat-shock protein structure

- Heat-shock proteins (HSPs) have been studied for their role in protecting cells from high temperature and stress conditions.
- HSPs have been classified into six major families based on their molecular masses such as HspH (Hsp110), HspC (Hsp90), HspA (Hsp70), DNAJ (Hsp40), HspB (small HSPs), and the chaperonin families HspD/E (Hsp60/Hsp10) and CCT (TRiC).
- The inducible HSP expression is regulated by the heat-shock transcription factors.
- The structure of Hsp70 includes an N-terminal ATPase domain (~44 kDa), a substrate binding domain (~18 kDa) and a C-terminal domain (~10 kDa).
- The conserved structure of cytosolic Hsp90 has an N-terminal domain containing the cochaperone-binding motif and an ATP and drug-binding site (e.g., geldanamycin and radicicol), an intermediate domain for binding to cochaperone and client proteins, and a C-terminal domain including a dimerization motif, a second drug-binding site and a conserved MEEVD pentapeptide.
- Most information on structure of Hsp60 is available from its prokaryotic homolog (i.e., GroEL). GroEL protein is composed of 14 identical subunits of 57 kDa, each forming two heptameric rings arranged back-to-back. Each GroEL monomer possesses three structural domains including apical, intermediate and equatorial (E1 and E2 subdomains).
- The members of small Hsp (sHsp) family have low molecular masses (~13–43 kDa) and contain a large N-terminal domain, a conservative α-crystallin domain (~90 residues), and a short C-terminal extension. The α-crystallin domain forms stable dimers that are the building blocks of the large sHsp oligomers.
- HSPs are often divided as the constitutive (cognate) and inducible forms.

Function of HSPs

- HSPs have been involved in different functions including chaperone activity, protein folding, apoptosis, autophagy and immunity.
- HSP families stimulate innate immunity through Toll-like receptors and scavenger receptors.
- HSP-mediated phagocytosis enhances the presentation of internalized antigens via the endocytic pathway in adaptive immune system.

Treatments

- HSPs have been used for development of prophylactic and therapeutic vaccines against a variety of diseases.
- Mutations in human sHSPs resulted in myopathies, neuropathies and cataract.
- Several studies demonstrated the relationship between HSPs and drug resistance as well as their use as a novel biomarker for detecting tumors in patients.
- Hsp90 inhibitors are important as therapeutic agents, especially anticancer drugs.
- HSPs (Hsp60, Hsp70, Hsp90)-harboring exosomes secreted by anticancer drug-treated human hepatocellular carcinoma cells (e.g., HepG2 cells) effectively induced HSP-specific natural killer (NK) cell cytotoxic responses and Granzyme B secretion, upregulated the expression of CD94 inhibitory receptor and downregulated the expression of CD69, NKG2D and NKp44-activating receptors.

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