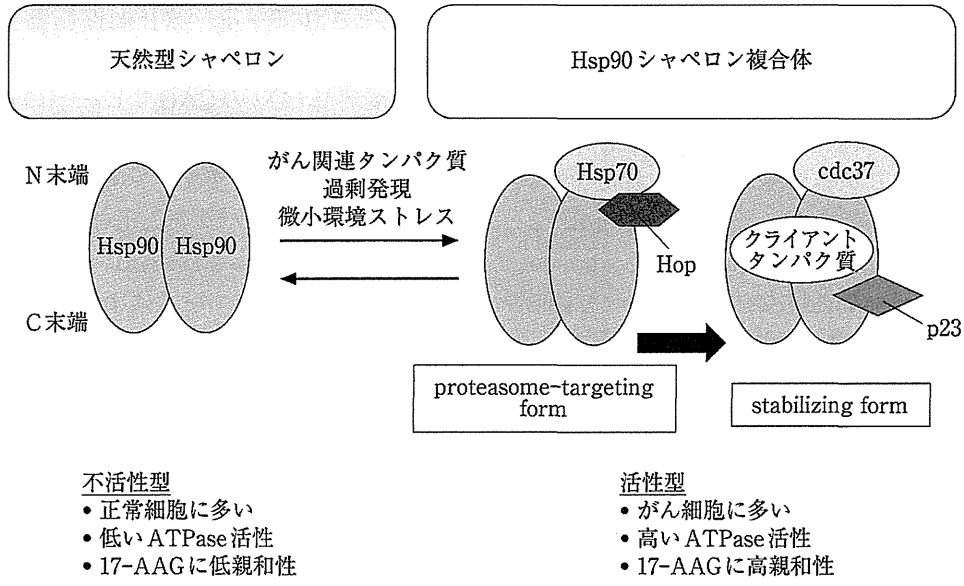

図2 正常細胞とがん細胞におけるHsp90の存在様式の差異

効率良くaddictedながん細胞を傷害でき，かつHsp90阻害剤耐性がん細胞の出現を抑制できるという他の抗がん剤にはない優れた特徴を有する．なお，Hsp90のクライアントタンパク質はPicard laboratoryによってupdateされているので，是非参照していただきたい[http://www.picard.ch/downloads/Hsp90interactors.pdf].

5 Hsp90シャペロン複合体

がん細胞由来のHsp90は，複数のコ・シャペロンと複合体を形成していることからHsp90シャペロン複合体と呼ばれている．このため腫瘍におけるHsp90では，正常細胞由来のHsp90と比較してATPase活性が高く維持されており，Hsp90阻害剤に対して100倍ほど親和性が高い[8]（**図2**）．また，変異型がん遺伝子産物はHsp90によるフォールディングをより必要とするため，Hsp90との結合親和性が正常のそれと比較して高いことが知られている．このため，正常組織に対する毒性を軽減することが可能であり，Hsp90阻害剤は有望な抗がん剤として臨床応用が期待されている．更に，Hsp90シャペロン複合体は，Hsp70，HOPが会合しているproteasome-targeting formとcdc37とp23が会合しているstabilizing formに分けることができる[3,5]．後述するHsp90阻害剤である17-AAGは，p23が会合した成熟Hsp90シャペロン複合体を，Hopが会合するproteasome-targeting formにshiftすることでクライアントタンパク質の分解を誘導する[9]．

6 Hsp90阻害剤の発見とその作用機序

GAは抗真菌剤のスクリーニングで見いだされたベンゾキノン系の低分子化合物であるが[10]，実験動物の固形腫瘍を縮小させることがわかり，抗がん剤としての応用が期待されるようになった．そしてGAに結合する細胞内タンパク質の精製・同定が行われた結果，Hsp90がその直接のターゲットであることが明らかにされた[11]．GAはHsp90のATP bindingポケットに結合してそのシャペロン機能を抑制する特異的な阻害剤であり，Hsp90依存性のクライアントタンパク質は，正常な構造を形成できずに活性が低下し，最終的にユビキチン-プロテアソーム系により分解される．GAは腎臓・肝臓に対する毒性が比較的強く臨床応用に適さなかったが，毒性の低い誘導体である17-allylamino-

17demethoxygeldanamycin(17-AAG), 17-dimethylamino-ethylamino-17demethoxygeldanamycin(17-DMAG)が見いだされ，現在臨床試験が行われている[12]．最近では，Hsp90のN末端側にATPが結合するという特徴を用いたスクリーニングにより，1-(2-phenol)-2-naphthol，5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosideなどが同定されている．実際，Hsp90阻害剤は，様々な種類のがんに対して抗腫瘍活性を示し，現在第1世代および第2世代のHsp90阻害剤は骨髄腫，乳がん，肺がん，前立腺がん，腎がんなどを対象に臨床試験(第1-第3相試験)が行われている[13]．

7 臨床試験が行われている新規Hsp90阻害剤

ganetispib(STA-9090)は，geldanamycinとは構造が異なる化合物であるが，Hsp90のATP結合ポケットに結合し，Hsp90の機能を抑制する[14,15]．ganetispibはHER2/neu，mutated EGFR，Akt，c-Kit，IGF-1R，PDGFRα，Jak1，Jak2，STAT3，STAT5，HIF-1α，CDC2，c-Met，Wilms' tumor 1のプロテアソームによる分解を促進することが明らかにされている．ganetispibは静脈内投与可能であり，現在，進行性非小細胞性肺がんに対して臨床試験(第2相)が実施されており，重度の副作用もなく，部分奏効を認めたと報告されている(2011年世界肺がん学会)．更にganetispibとtaxane系抗がん剤との併用で，抗腫瘍効果が増強するとの報告があり[15]，今後の臨床試験の結果が期待される．

8 神経変性疾患に対するHsp90阻害剤の効果

がん以外の臨床応用については，CAGリピートの異常によるポリグルタミン病についての報告がなされている．球脊髄性筋萎縮症(spinobulbar muscular atrophy: SBMA)は，アンドロゲン受容体(AR)のCAGリピートの過伸張による運動ニューロンの変性・細胞死が原因である．祖父江らはこの変異アンドロゲン受容体がクライアントタンパク質としてHsp90と結合していることを示し，17-AAGを投与すると，変異ARがプロテアソームによる分解を受けることにより，運動ニューロンの変性が抑制され，症状が改善することを示した[16]．この際，17-AAG処理により，Hsp90-ARシャペロン複合体はp23の会合する安定型から，HOPの会合するプロテアソーム標的型へと変換されることを示している[9]．

おわりに

結晶解析の発達によりHsp90の構造とコ・シャペロンとの協働作用の分子メカニズムが明らかにされ，Hsp90の分子シャペロン機能が飛躍的に解明されている．このことは現在の研究の中心であるATPaseドメインを標的としたHsp90阻害剤に加えて，Hsp90とコ・シャペロンの分子会合を阻害する分子標的薬もまた有用である可能性を示す．一方，Hsp90のクライアントタンパク質は現在でも増加しており，最近では細胞内の自然免疫受容体であるNLR[Nod(nuclear oligomerization domain)-like receptor]もその1つであることが報告された．将来，Hsp90を標的にした慢性炎症や自己免疫疾患に対する免疫応答制御法の開発につながる期待を抱かせる．しかし，Hsp90が構造の異なるクライアントタンパク質をいかにして認識し，フォールディングするのか，更に種々のコ・シャペロンとの結合・解離の分子機構は依然アンフォールディング(未成熟)のままである．この未開の分子に立ち向かう研究の広がりが画期的な治療法につながる可能性は大いにある．

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Heat-shock proteins as endogenous ligands building a bridge between innate and adaptive immunity

There has been growing evidence that heat-shock protein (HSP) functions as an endogenous immunomodulator for innate and adaptive immune responses. Since HSPs inherently act as chaperones within cells, passive release (e.g., by cell necrosis) and active release (including release by secretion in the form of an exosome) have been suggested as mechanisms of HSP release into the extracellular milieu. Such extracellular HSPs have been shown to be activators of innate immune responses through Toll-like receptors. However, it has also been suggested that HSPs augment the ability of associated innate ligands such as lipopolysaccharides to stimulate cytokine production and dendritic cell maturation. More interestingly, a recent study has demonstrated that innate immune responses elicited by danger signals were regulated spatiotemporally and that can be manipulated by HSPs, thereby controlling immune responses. We will discuss how spatiotemporal regulation of HSP-chaperoned molecules within antigen-presenting cells affects adaptive immunity via antigen cross-presentation and innate immune responses. Precise analysis of HSP biology should lead to the establishment of effective HSP-based immunotherapy.

KEYWORDS: cross-presentation danger signal dendritic cell heat-shock protein Toll-like receptor

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Heat-shock proteins (HSPs), highly conserved across species, are generally considered to be intracellular proteins that have protective functions in situations of cellular stress [1]. A wide variety of stressful stimuli such as heat shock, UV radiation, and viral or bacterial infections induce a substantial increase in intracellular HSP synthesis. The main functions of HSPs are to act as chaperones of nascent or aberrantly folded proteins [1,2]. HSPs have also been shown to have important functions in the mammalian immune system. Srivastava's group first identified the endoplasmic reticulum (ER)-resident HSP gp96 as a tumor antigen [3,4]. Immunization of mice with gp96 isolated from tumors induced an antitumor immune response through the induction of tumor-specific cytotoxic T lymphocytes (CTLs). This immunogenicity is based on antigenic peptides that are associated with gp96 molecules, and peptide-deprived HSP complexes lose their specific immunogenicity. Following these observations, Srivastava's group further demonstrated that immunization with tumor-derived Hsp70 and Hsp90 also elicited tumor-specific CTL responses [5,6]. In particular, they showed that Hsp70 bound tumor-specific antigenic peptides because Hsp70 treated with ATP released bound peptides, resulting in loss of their immunogenicity [5]. Noessner *et al.* also demonstrated that tumor-derived Hsp70 bound a tumor antigen (tyrosinase) peptide [7]. Moreover, the role of extracelluar HSPs in the stimulation of innate immunity has drawn much attention in recent years. We will discuss the current view of this unique feature of HSPs in the regulation of innate and adaptive immune responses.

HSPs as key players of the immune system

Recently obtained evidence indicates that extracellular HSPs play an important role in the induction of innate immune responses [2]. Since HSPs do not have a canonical signal sequence, it has been suggested that HSPs may be released via an active secretion mechanism or from cells undergoing necrosis [2,8]. The resultant extracellular HSPs may then interact with dendritic cell (DCs) or macrophages, resulting in the activation of innate immune responses including maturation of DCs and secretion of proinflammatory cytokines and chemokines through Toll-like receptor (TLR) activation [9,10]. This unique feature of certain HSPs is termed 'chaperokine', which describes that extracellular HSPs act as both chaperones and cytokines [11]. By contrast, regarding the adaptive immunity, HSPs have another unique feature acting as a cross-presentation inducer. DCs have a unique ability to take up, process and present exogenous antigens in association with MHC class I molecules, termed cross-presentation [12]. The cross-presentation

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plays a pivotal role in priming antigen-specific naive T-cell responses to tumor cells and virus-infected cells that cannot access the classical pathway for MHC class I presentation. It has been shown that HSPs can be released in the form of complexes with antigenic peptides and that these HSPs–peptide complexes are taken up by antigen-presenting cells (APCs) such as DCs through specific receptors expressed on APCs, leading to cross-presentation [4,8,13]. Thus, extracellular HSPs act as chaperokines for activation of innate immunity as well as enhancers for adaptive immunity of antigen-specific T-cell induction (summarized in TABLE 1).

These findings led to the idea that APCs bear HSP-specific receptors on the cell surface. Following the identification of CD91/low-density lipoprotein receptor-related protein-1 (LRP-1) as a gp96 receptor [14], many receptors including members of the TLR and scavenger receptor (SR) families have been shown to be HSP receptors.

Considering the establishment of HSP-based cancer immunotherapy, it is very important to develop the orchestrated link between innate and adaptive immunity via specific HSP receptors. Therefore, we next overview the HSP receptors expressed on APCs.

HSP receptors

HSPs interact with a range of receptors expressed on target cells. These receptors can be divided into two groups: TLRs and SRs [15]. TLRs are major pattern-recognition receptors (PRRs) and 11 have been identified in mammals. Two TLRs, TLR2 and TLR4, have been demonstrated to function as receptors for Hsp60, Hsp70 and gp96, leading to NF-κB activation [16–18]. In addition, the cell-surface protein CD14 required for lipopolysaccharide (LPS)-mediated TLR4 activation, has been shown to be required for Hsp70-mediated induction of cytokines TNF-α, IL-1β and IL-6 [10]. Lehner *et al.* showed that immunological consequences of Hsp72 can be localized to specific domains of the Hsp72 molecule. The C-terminal portion of Hsp72 (amino acids 359-610) stimulates production of chemokines, IL-12, TNF-α and NO, induces Th1 polalization and stimulates the maturarion of DCs. The N-terminal ATPase domain (amino acids 1-358) largely lacks these functions [19]. Wheeler *et al.* have also demonstrated that the C-terminal region of Hsp72 serves as an activator for macrophages to produce TNF-α. These effects did not seem to result from LPS contamination [20]. However, some studies have suggested that these interactions between Hsp70 and TLR are not likely to be exerted through the direct binding of Hsp70 to CD14, TLR2 or TLR4, as cells stably transfected with CD14, TLR2 or TLR4 do not bind avidly to Hsp70 [21]. These findings suggest that low-affinity interactions may be involved in TLR activation by Hsp70. A previous study showed that TLR activation by Hsp60 requires the internalization of the Hsp60; therefore, experiments using cells with simple TLR gene overexpression may thus be inadequate to assess direct HSP–TLR binding [18,22].

SRs constitute the other family of PRRs. These are receptors for chemically modified

Table 1. Classification and function of major mammalian heat-shock proteins in immunological fields.

Family	HSPs	Intracellular localization	Cross-presentation	Induction of inflammatory cytokine
Small HSP	Hsp25/27/28	Cytosol	ND	ND
Hsp40	Hsp40	Cytosol	ND	ND
Hsp47	Hsp47	ER	ND	ND
Hsp60	Hsp60	Mitochondria	ND	Enhance [19]
Hsp70	Hsp70	Cytosol	Enhance [5,6,7,13]	Enhance [9,11,16], inhibit [81]
	Hsc70	Cytosol	ND	ND
	mtHsp70	Mitochondria	ND	ND
	BiP (Grp78)	ER	Enhance [82]	ND
Hsp90	Hsp90	Cytosol	Enhance [74,75]	Enhance [17]
	Gp96/Grp94	ER	Enhance [14,23]	Enhance [17]
Hsp100	Hsp105/Hsp110	Cytosol	Enhance [83]	Enhance [84]
	ORP150/Grp170	ER	Enhance [80,85]	ND

ER: Endoplasmic reticulum; HSP: Heat-shock protein; ND: Not determined.

forms of lipoproteins, including oxidized and acetylated low-density lipoproteins. The SR family is divided into eight subclasses (A to H), and many receptors belonging to this family are expressed on the surface of APCs. The oxidized low-density lipoprotein-binding protein CD91/LRP has been shown to be a common receptor for Hsp60, Hsp70, Gp96 and calreticulin [23]. However, Theriault showed that the difference of Hsp70 binding to CD91-positive and -negative cells was minimal [21]. Therefore, Hsp70 binding to CD91 may be a low-affinity interaction or may be indirect. It has been demonstrated that Hsp70 can interact with at least three members of the SR family, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) [24], SR expressed by endothelial cells-1 (SREC-1) [25], and fasciclin, EGF-like, laminin-type EGF-like and link domain-containing SR-1 (FEEL-1) [25]. Hsp70 can be bound at high affinity by these SRs and internalized. Both Hsp90 and Hsp60 can also bind to LOX-1. In addition, gp96 and calreticulin show significant affinity to SR-A1 and SREC-1 and are internalized by these receptors [26,27]. However, HSP (gp96) binding to SR-A1 is immunosuppressive [28], whereas LOX-1 mediates Hsp70 immunogenicity and antigen presentation [24]. Therefore, detailed studies of each receptor–HSP interaction are essential to determine the effects of HSPs on immune responses.

Mechanism of HSP release from cells

As described above, immunization with HSP–peptide complexes elicits antitumor immune responses via cross-presentation by APCs. Do immune responses induced by extracellular HSP-mediated antigen cross-presentation actually take place *in vivo*? Because HSPs are inherently intracellular proteins, some mechanism for the release of endogenous HSPs into the extracellular space must exist. However, HSPs are not secreted via the classical pathway because their sequences encode no secretion leader signals. In fact, as described later, it has been shown that the export of HSPs to the extracellular space could not be blocked by typical inhibitors of the ER–Golgi pathway, such as brefeldin A. Currently, two mechanisms are considered to result in the release of HSPs from cells: passive release mechanisms such as necrotic cell death caused by exposure to hypoxia, severe trauma, surgery and lytic virus infection [29]; and active release mechanisms involving nonclassical protein release pathways [8,30–32].

Passive release mechanism

Release of intracellular proteins involves cell lysis and this may occur in pathological conditions that give rise to necrosis [29]. Basu and coworkers reported that HSPs, including gp96, Hsp90, Hsp72 and calreticulin, are released from necrotic cells but not from apoptotic cells [17]. Necrotic cell death results in the release of intracellular contents into the extracellular space, thereby liberating HSPs. Their findings make cell necrosis an attractive explanation for the mechanism by which HSPs are released into the extracellular milieu as an intrinsic immunological messenger termed endogenous danger signal as described later.

Active release mechanism

An active release mechanism has been suggested as an additional mechanism to the passive release hypothesis. Asea *et al.* showed that IFN-γ and IL-10 induce the active release of constitutively expressed Hsp70 (Hsc73) as well as Hsp72 from tumors under conditions that will not induce cell death [30]. Since IFN-γ and IL-10 are thought to exist at high concentrations within inflammatory foci, these cytokines may mediate the active release of Hsc73 and Hsp72 *in vivo* [31]. Moreover, Asea's group showed that whereas some extracellular Hsp72 could be found as free Hsp72, a proportion of extracellular Hsp72 was released within exosomes [31,32]. Exosomes are internal vesicles of multivesicular bodies released into the extracelluar milieu upon fusion of an multivesicular bodies with the cell surface. Additionally, they showed that Hsp72 was released by a nonclassical protein transport pathway and that intact surface membrane lipid rafts were required for efficient stress-induced Hsp72 release. Mambula *et al.* also demonstrated that a prostatic cancer cell line secreted Hsp72 via an endolysosomal pathway [8]. Furthermore, Fleshner *et al.* demonstrated another possible mechanism for Hsp72 release under the condition of psychological stress [33]. They proposed that activation of the sympathetic nervous system by stimuli such as stressor exposure results in the release of norepinephrine and subsequent activation of α1-adrenergic receptors. Stimulation of α1-adrenergic receptors results in an increase in intracellular Ca^{2+}, which may stimulate the release of exosomes containing Hsp72. More recently, Vega *et al.* [34] and De Maio [35] have demonstrated that Hsp72 is inserted into the plasma membrane of cells after stress, which may be formed by inverse evagination. It was also shown that

Hsp72 was released into the extracellular space in a membrane-associated form (i.e., exosome) that could act as a danger signal to activate macrophages. Strikingly, activation of macrophages by the membrane-associated form of Hsp72 was highly effective than that by the free recombinant Hsp72. This robust effect is likely to be due to the high concentration of Hsp72 within the vesicle (exosome).

Taken together, the results of these studies suggest that the active release hypothesis is an important mechanism by which Hsp72 is released into the extracellular milieu.

Extracellular HSPs act as endogenous danger signals

The 'danger theory' postulates that the host releases endogenous signals that are derived from stressed or damaged cells, capable of stimulating immunity [36]. Accumulating evidence indicates that extracellular HSPs fulfil the criteria of an endogenous danger signal.

HSPs are chaperokines

Preparations of HSPs such as Hsp60, Hsp70, Hsp90, gp96 and Hsp110 purified from a variety of sources including bacterial and mammalians, as well as recombinant bacterial and human products, have been reported to be potent activators of innate immunity, indicating that HSPs act as danger signals. Specifically, these HSP preparations have been reported to stimulate the production of proinflammatory cytokines such as TNF-α, IL-1, IL-6 and IL-12 and the release of nitric oxide (NO) and C-C chemokines by monocytes, macrophages and DCs [10,37–40]. HSPs have also been reported to induce the maturation of DCs, as demonstrated by the upregulation of MHC class I and II molecules and costimulatory molecules such as CD80, CD86 and CD40 [40,41]. Moreover, Chen *et al.* have demonstrated that heat stress induced the release of various HSP from tumor cells, which, in turn, activated tumor cells to produce chemokines for chemoattraction of DCs and T cells via the TLR4 signaling pathway [42]. Thus, it has been proposed that, through their cytokine-like functions, HSPs serve as 'chaperokines' to the host's immune system at sites of tissue injury or stress where HSPs are released into extracellular spaces. The discovery of extracellular biological actions of HSPs as chaperokines is very attractive and the chaperokine theory is interesting with regard to extracellular HSPs as a cause of sterile inflammation.

HSPs augment the action of associated microbial products

There is some concern that these chaperokine activities might have been due to microbial contamination of HSP preparations. The representative microbial product LPS is a strong stimulus of innate immune signaling. Wallin *et al.* reported that highly purified murine liver Hsp70 had no cytokine effects even at concentrations as high as 200–300 mg/ml [43]. Thus, some HSPs have been shown to bind microbial products very efficiently and thus augment the biological actions of microbial products. Habich *et al.* demonstrated that Hsp60 bound LPS tightly and that Hsp60-bound LPS, but not Hsp60 itself, was responsible for the observed cytokine effects of Hsp60 preparation [44]. More interestingly, Hsp60-bound LPS was more potent than LPS in inducing cytokine production. Reed *et al.* showed that gp96 binds endotoxin in a high affinity, saturable and specific manner. The same study demonstrated that low (<0.27 EU endotoxin/mg protein) endotoxin preparations of gp96 do not stimulate NF-κB activation or NO release in macrophages [45]. In addition, Wager *et al.* showed that gp96 bound lipid-based TLR ligands such as palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam_3Cys; a TLR2 ligand) and LPS (TLR4 ligand). Binding of Pam_3Cys and LPS to gp96 enhanced their ability to induce the activation and maturation of DCs [46]. Thus, there may be cooperation between gp96 and TLR ligands, and the former may amplify the effects of the latter. These results suggested that if 'chaperoned by a certain HSP', LPS at very low concentrations could induce cytokine production. In this context, HSP augments immune responses against molecules chaperoned by HSPs but not against HSPs themselves. In a clinical setting, this unique character of HSPs may contribute to the *in vivo* recognition of Gram-negative bacterial infection by binding to LPS and augmenting the host defense system via TLR4. Although further studies are necessary to determine whether the reported cytokine-producing effect is a result of HSPs or contamination of TLR ligands, there is no doubt that HSPs play important roles in eliciting immune responses by acting as danger signals via HSPs themselves and/or HSP-associated molecules.

Cell surface-expressed HSPs elicit immune responses

To avoid the problem of microbial contamination, a number of studies have been conducted on transgenic expression of HSPs on the cell

surface. It has been demonstrated that cell-surface HSPs can activate immune responses. Under these conditions, contamination by exogenous molecules such as LPS is unlikely. Zheng *et al.* demonstrated that cell surface expression of gp96 on tumor cells induced efficient T-cell priming and tumor rejection *in vivo* [47]. Likewise, Chen *et al.* showed that transgenic expression of Hsp70 on the tumor cell surface elicited antitumor immunity, and immunization of mice with these tumor cells led to induction of tumor-specific cytotoxic T cells [38]. In addition, transgenic expression of gp96 on the cell surface leads to *in vivo* activation of DCs and the development of lupus-like systemic autoimmune disease in mice [48,49]. Vaccination of mice with gp96-secreting fibroblasts leads to *in vivo* activation of $CD11b^+$ and $CD11c^+$ APCs [50]. These studies have demonstrated that cell-surface HSPs are capable of activating the immune system *in vivo*. However, it remains unclear whether the observed *in vivo* effects are direct effects of HSPs themselves or effects via HSP-associated molecules.

Getting a grip on the chaperokine world

It must be emphasized that HSP purification procedures should be carried out to the highest standards of molecular chaperone purification as described by Pockley *et al.* [51]. In addition, a further direction of this field will be to identify the receptor binding domain of each HSP. More importantly, x-ray crystallographic analysis may reveal the mode of HSP-receptor binding. From the biological point of view, as an endogenous danger signal, under the condition of bacterial or viral infection, damaged cell-derived extracellular HSPs may bind microbial products such as LPS, lipoproteins, bacterial DNA and viral RNA in the inflamed milieu, leading to activation of TLR- and non-TLR-mediated innate immune responses. This nascent but very attractive field of biology should be further developed through careful experiments.

Endogenous danger signals & sterile inflammation concomitant with immune responses

In addition to recognizing pathogen-associated molecular patterns, the immune system has evolved to recognize endogenous danger signals, called damage-associated molecular patterns (DAMPs), many of which are released by dying or necrotic cells and contribute to 'sterile inflammation' in a noninfectious sterile setting. Furthermore, the recognition of DAMPs can activate the innate immune system *in vivo*. DAMP molecules comprise a structurally and sequence-diverse family of endogenous molecules, generally intracellular and hidden by the plasma membrane, that are often released from necrotic cells. It has been demonstrated that uric acid [22], DNA and more specifically unmethylated CpG-rich DNA regions, high-mobility group box 1 (HMGB1) [52,53], Sin3A-associated protein 130 [54], IL-1α [55], IL-33 [55,56], S100 proteins [57] and HSPs act as DAMPs [58]. However, a recent study has suggested that highly purified HMGB1 does not induce significant amounts of proinflammatory cytokines. The capacity of HMGB1 to bind other molecules may be the underlying basis for these observations [59]. Thus, some of these DAMPs have the ability to interact with other molecules, including DNA, RNA, IL-1β and LPS, and also nucleosomes that augment or modify the function of DAMPs themselves. As mentioned previously, HSPs can also bind a number of endogenous as well as pathogen-associated exogenous molecules and enhance the cytokine effects of these molecules. Recently, we have shown that extracellular Hsp90 can enhance the self-DNA-mediated type I IFN production by plasmacytoid DCs (pDCs) and conventional DCs (cDCs). In the next section we will discuss how Hsp90 is able to change the destination of associated molecules via spatiotemporal regulation.

Hsp90-mediated spatio-temporal regulation of CpG-oligodeoxynucleotide & activation of innate immunity

In contrast to the idea that HSP itself acts as an endogenous danger signal, we have a working hypothesis that HSP empowers the chaperoned innate ligands to activate innate immune response [60]. Unmethylated CpG dinucleotides within certain sequence contexts (CpG motifs) are recognized by TLR9, which is expressed primarily by pDCs and B cells, resulting in a large amount of IFN-α production [61,62]. We have shown that murine and human pDCs pulsed with an Hsp90-CpG-A oligodeoxynucleotide complex produce a larger amount of IFN-α than that in the case of CpG-A alone (FIGURE 1A). Furthermore, unlike human DCs, murine cDCs express both TLR7 and TLR9, although the expression levels are low compared with those in pDCs. We then showed the ability of Hsp90 to target chaperoned CpG-A to murine cDCs, resulting in IFN-α production (FIGURE 1B). The

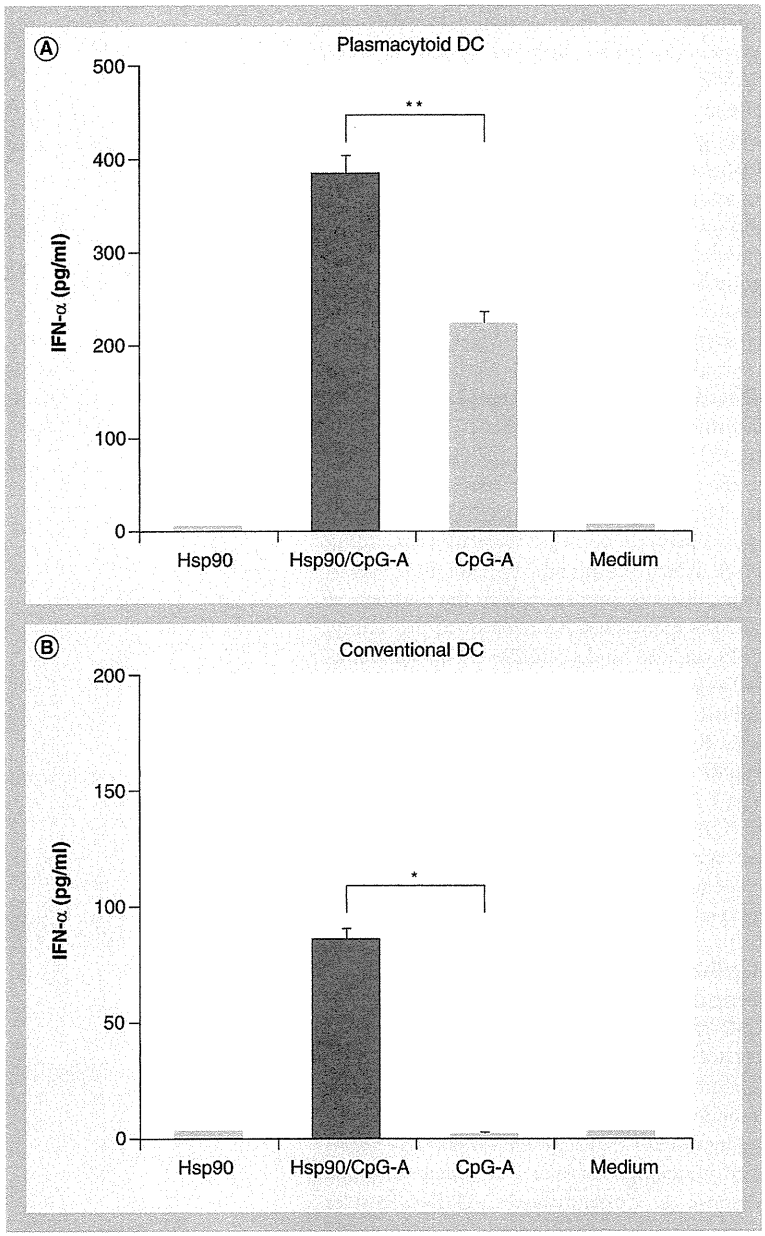

Figure 1. Hsp90–CpG-A complexes enhance induction of IFN-α by murine dendritic cells. (A) Hsp90–CpG-A complexes induce IFN-α production by plasmacytoid DCs to a level twofold higher than that induced by CpG-A alone. **(B)** Conventional DCs produce IFN-α when stimulated with Hsp90-CpG-A complex but not when stimulated with CpG-A alone. Hsp90 alone does not have any effect on IFN-α production. Data are presented as means + standard error of the mean of triplicate wells.
*$p < 0.0005$, **$p < 0.001$; paired student t-test.
DC: Dentric cell.

observed IFN-α production was shown to be TLR9-dependent. We found that Hsp90-chaperoned CpG-A was localized and retained within static early endosomes for longer periods in cDCs, thereby eliciting TLR9 signaling for IFN-α production, but not inflammatory cytokines such as IL-6 and TNF-α. By contrast, CpG-A alone moved into late endosomes and lysosomes within cDCs. Interestingly, not only CpG-A but also CpG-B could stimulate the TLR9 signaling within static early endosomes, resulting in the production of IFN-α. Thus, extracellular Hsp90 has the ability to direct associated CpGs into static early endosomes, leading to interferon regulatory factor 7 activation and IFN-α production.

Why, however, are DNA–Hsp90 complexes retained in early endosomes but not in late endosomes or lysosomes in DCs? We found that endocytosed CpG-A–Hsp90 complexes were selectively transferred into Rab5$^+$, early endosomal antigen (EEA)-1$^+$ static early endosomes. Very recently, Lakadamyali *et al.* have shown that early endosomes are comprised of two distinct populations, a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome and a static population that matures much more slowly [63]. Cargos destined for degradation, including low-density lipoprotein, EGF and influenza virus, are internalized and targeted to the Rab5$^+$, EEA1$^-$ dynamic population of early endosomes, thereafter trafficking to Rab7$^+$ late endosomes. By contrast, the recycling transferrin is delivered to Rab5$^+$, EEA1$^+$ static early endosomes, followed by translocation to Rab11$^+$ recycling endosomes. They also found that cargos trafficked into these static early endosomes were retained for longer periods and not translocated into late endosomes and lysosomes. Thus, our observation that the CpG-A–Hsp90 complex was retained in static early endosomes, leading to sustained activation of DCs and IFN-α production, is consistent with their findings (Figure 2).

Hsp90 as a possible accelerator for autoimmune diseases

pDCs normally do not respond to self-DNA, which may reflect the fact that viral/bacterial DNA sequences contain multiple CpG nucleotides that bind and activate TLR9, whereas mammalian self-DNA contains fewer such motifs, which are most likely masked by methylation. Recent evidence, however, suggests that self-DNA has the potential to trigger TLR9 but may fail to do so because it fails to access the TLR9-containing endolysosomal compartments. One of the mechanisms to which this effect is attributed is the fact that DNase easily and rapidly breaks down extracellular DNA, thereby hampering self-DNA localization into

endocytic compartments. The importance of this mechanism in preventing autoimmune responses has been shown by the fact that mice deficient in DNase II develop systemic lupus erythematosus (SLE)-like syndrome [64]. Recently, it has been demonstrated that pDCs do sense and respond to self-DNA in human autoimmune diseases. Therefore, we examined whether Hsp90 targets self-DNA into static early endosomes, resulting in IFN-α production by human pDCs [60]. Upon Hsp90-mediated enforced endosomal translocation, both human self-DNA and CpG-ODN could activate DCs via TLR9 to produce IFN-α. Previous studies have demonstrated the presence of autoantibodies to Hsp90 [65,66] and enhanced expression of Hsp90 in peripheral blood mononuclear cells of patients with active SLE [67,68], suggesting a role of Hsp90 in the pathogenesis. In addition, Hsp90 has been shown to localize both in the cytoplasm and nucleus [69]. Moreover, under stressful conditions, it has been shown that cytosolic Hsp90 translocates to the nucleus [70]. This suggests that Hsp90 may bind self-DNA within the nucleus. When cells undergo necrosis, self-DNA associated with endogenous Hsp90 could be released into the extracellular space and might trigger IFN-α production by pDCs. Our findings support the idea that Hsp90, an endogenous danger signal molecule found in sera of SLE patients, might be the key mediator of pDC activation in SLE. Thus, Hsp90 may activate innate immunity to self-DNA by forming a complex with self-DNA that is delivered to and retained within early endocytic compartments of pDCs to trigger TLR9 and induce IFN production. Thus, we determined a fundamental mechanism by which pDCs sense and respond to self-DNA coupled with Hsp90. Our data suggest that, through this pathway, pDCs drive autoimmunity in autoimmune diseases. Several host factors other than Hsp90 that can convert self-DNA into a trigger of DC activation have been reported. Endogenous cationic antimicrobial peptide LL37 (also known as CAMP) [71], autoantibodies [72] and HMGB1 [59] have been demonstrated to do so by forming a complex with host-derived DNA. Together, these findings indicate that the ability of some of DAMPs to convert self-DNA into a trigger of high levels of IFN-α production depends on the capacity of DAMPs to concentrate and retain DNA in static early endosomes, thus enabling the selective and sustained activation of early endosomal TLR9.

Cross-presentation mediated by HSP: pivotal role of Hsp90

HSPs inherently act as molecular chaperones, and in our opinion, from an immunological point of view, the most characteristic finding is that HSPs can bind antigenic peptides within cells. Therefore, immunization with a HSP–antigenic

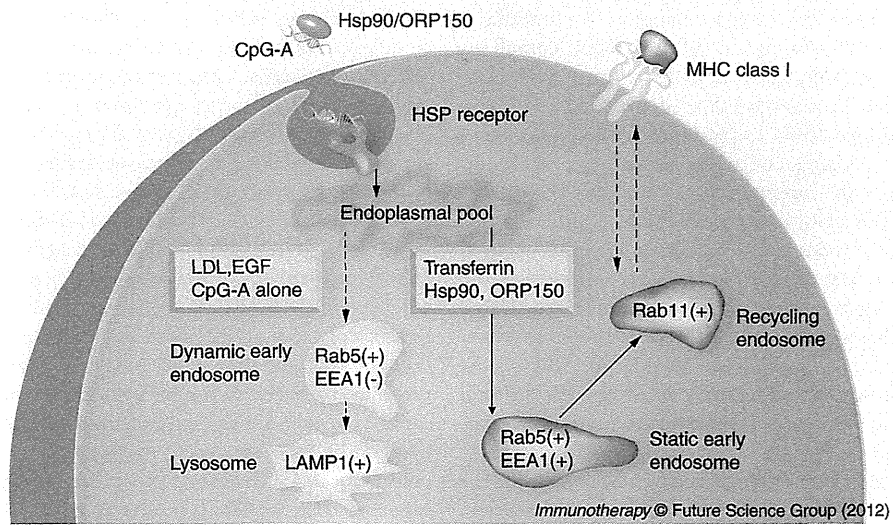

Figure 2. Extracellular Hsp90/ORP150 targets chaperoned molecules into static early endosomes. Early endosomes are comprised of static early endosomes (Rab5$^+$ and EEA-1$^+$) and dynamic early endosomes (Rab5$^+$ and EEA-1$^-$). Hsp90/ORP150-antigen or Hsp90–CpG-A complexes are preferentially targeted and retained in static early endosomes of DCs, leading to efficient antigen cross-presentation as well as sustained activation of Toll-like receptor 9 and IFN-α production.
Hsp: Heat-shock protein; LDL: Low-density lipoprotein.

peptide complex elicits antigen-specific cytotoxic T-cell responses. Therefore, extracellular HSP can deliver associated antigens into the MHC class I presentation pathway of antigen-presenting cells, a process called cross-presentation, thus inducing antigen-specific $CD8^+$ T-cell responses. However, the underlying mechanism for introduction of an exogenous antigen into a cross-presentation pathway remains unclear, and the precise mechanism for intracellular antigen translocation and the processing pathway have not been fully elucidated. Recent evidence indicates that exogenous antigens can be processed through at least two distinct pathways, one involving access of exogenous antigens to the classical MHC class I loading pathway (TAP-dependent) and the other involving unconventional post-Golgi loading of MHC class I molecules in endocytic compartments (TAP-independent) [73]. One or both of these pathways can contribute to cross-presentation depending on the source of exogenous antigens such as soluble proteins, immune complexes or peptides chaperoned by HSPs.

We first demonstrated that extracellular Hsp90-peptide complexes generated *in vitro* were efficiently cross-presented by DCs and recognized by peptide-specific CTLs via a TAP-independent 'endosome-recycling' pathway because DCs derived from TAP-knockout mice were fully functional in cross-presentation of an Hsp90–peptide complex [74]. Next, we showed that cross-presentation of extracellular Hsp90–ovalbumin (OVA) protein complexes to specific $CD8^+$ T cells involved both classical proteasome–TAP-dependent and TAP-independent endosome-recycling pathways [75]. Using confocal microscopy, we found that the internalized extracellular Hsp90 and OVA colocalized with cytosolic proteasomes. To investigate whether endogenous Hsp90 was responsible for cross-presentation of the exogenous Hsp90–OVA complex, we treated DCs with the Hsp90-specific inhibitor radicicol in a cross-presentation assay. The results showed that treatment of DCs with radicicol did not affect the cross-presentation of exogenous Hsp90-OVA, suggesting that endogenous Hsp90 might not be responsible for the exogenous Hsp90-mediated cross-presentation. Strikingly, when an anti-Hsp90 monoclonal antibody was introduced into DCs, the colocalization of internalized Hsp90-chaperoned OVA and proteasomes was abolished, resulting in inhibition of TAP-dependent cross-presentation of OVA. Thus, extracellular Hsp90 may play a pivotal role in the translocation of chaperoned antigens for proteasomal degradation in the cytosol. By contrast, a high concentration of soluble OVA (200 mg/ml) was cross-presented by DCs, although the cross-presentation was considerably less efficient than that of a Hsp90–OVA complex (20 μg/ml of OVA). Interestingly, this cross-presentation was radicicol-sensitive, indicating that endogenous Hsp90 played an important role in the cross-presentation of exogenous OVA. Udono *et al.* have elegantly demonstrated that endogenous Hsp90α was required for cross-presentation of exogenous OVA using DCs derived from Hsp90α-knockout mice [76]. Their findings also suggest that endogenous Hsp90, as well as exogenous Hsp90, might help the exogenous Hsp90–OVA complex translocate into cytosol at the cytosolic face for cross-presentation (FIGURE 3). By contrast, OVA chaperoned by Hsp90 was not presented by MHC class II molecules *in vitro* or *in vivo*, although the antigen was exogenously loaded onto DCs. To confirm these observations, we investigated the destination of Hsp90–OVA complexes and soluble OVA after its uptake in DCs, using confocal laser microscopy. Hsp90-chaperoned OVA was observed to colocalize with early endosomes, recycling endosomes, and proteasomes but not lysosomes. By contrast, soluble OVA was detected in early endosomes to lysosomes but not in recycling endosomes, ER or proteasomes. These findings indicated that Hsp90–OVA complexes and soluble OVA were sorted into different organelles. Thus, extracellular Hsp90 might be essential for translocation of chaperoned antigens from the extracellular milieu into the cytosol, resulting in proteasomal degradation for cross-presentation. These results have revealed a novel mode of involvement of Hsp90 in antigen presentation by DCs: exogenous Hsp90 preferentially introduces the chaperoned antigen into the MHC class I pathway, resulting in efficient cross-presentation. Since there is a classical paradigm that extracellular antigens are presented by MHC class II molecules, it seemed significant to find that Hsp90 changed the destination of the associated antigen on antigen presentation. Calderwood's group has recently reported that the SREC-1 acts as an Hsp90 receptor for cross-presentation on DCs [77]. As described previously, Lakadamyali *et al.* demonstrated that early endosomes are comprised of two distinct populations: a dynamic population that matures rapidly toward the late endosome and lysosome, and a static early endosome that

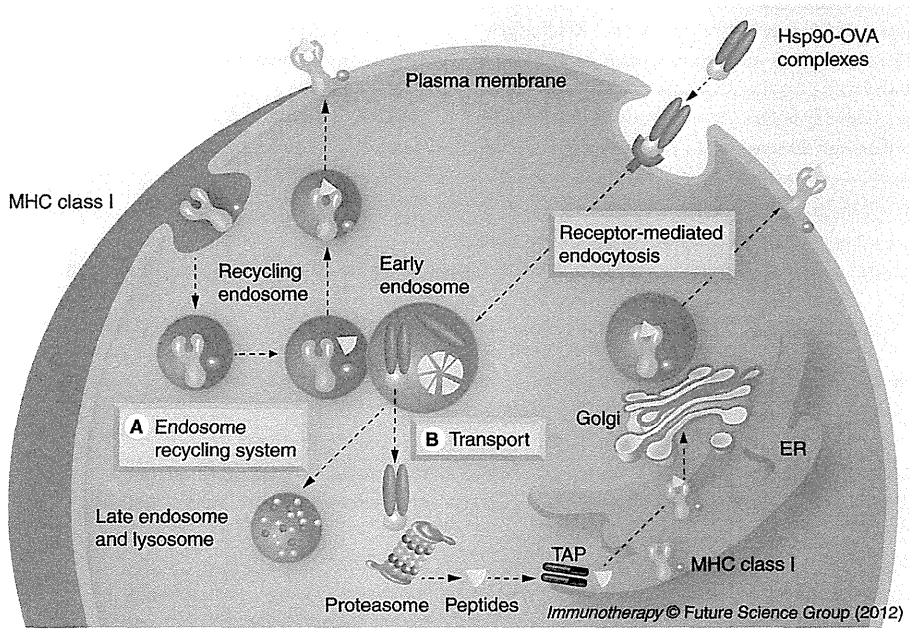

Figure 3. Pathway for Hsp90-antigen complex-mediated cross-presentation by DCs.
Internalized Hsp90-antigen complexes through receptor-mediated endocytosis follow two distinct MHC class I pathways. **(A)** Internalized antigens chaperoned by Hsp90 are processed by endosomal peptidases such as cathepsin S and are loaded in the endocytic pathway onto MHC class I molecules that are recycled from the plasma membrane (TAP-independent pathway). **(B)** Alternatively, internalized Hsp90-antigen complexes are translocated to the cytosol and are degraded by the proteasome. Resultant peptides are imported into the ER in a TAP-dependent fashion and are loaded onto newly synthesized MHC class I molecules.
ER: Endoplasmic reticulum; OVA: Ovalbumin.

matures much more slowly [63]. Interestingly, Burgdorf *et al.* demonstrated that a mannose receptor introduces exogenous OVA specifically into an EEA-1$^+$, Rab5$^+$ static early endosomal compartment for subsequent cross-presentation [78,79]. These observations are consistent with dynamics of extracellular Hsp90 demonstrated by us. By contrast, OVA endocytosed by a SR did not colocalize with EEA-1; instead, it colocalized with LAMP-1 in the lysosome as shown here, leading to presentation in the context of MHC class II molecules. Thus, we expect that Hsp90-specific receptors such as SREC-1 might introduce the Hsp90–OVA complex into the static early endosome for cross-presentation. Furthermore, we have recently shown that an ER-resident Hsp70 family member, oxygen-regulated protein 150 (ORP150), localized to static early endosomes after endocytosis, leading to antigen cross-presentation when pulsed onto DCs [80]. These CTLs induced by immunization with Hsp90/ORP150 peptide complexes could inhibit established tumor growth, indicating that Hsp90/ORP150 peptide complexes act as cancer vaccines. Importantly, the Hsp90/ORP150-mediated cross-presentation pathway for exogenous peptides has been shown to be an endosome-recycling pathway, not a conventional TAP-dependent pathway. Targeting static early endosomes is the key feature of Hsp90/ORP150 for inducing innate as well as adaptive immune responses. These findings provide a rationale for the development of Hsp90-based vaccination strategies for cancer as well as viral immunity.

Future perspective

Can all HSPs target chaperoned molecules to static early endosomes? It has been shown that Hsp90 and ORP150 could be targeted to static early endosomes after endocytosis, leading to antigen cross-presentation when pulsed onto DCs. Whether this is the case for other HSPs, however, remains to be determined. More importantly, the HSP receptor responsible for targeting to static early endosomes should be clarified. Elucidation of the molecular basis for sorting HSPs to static endosomes will also be necessary to establish HSP-based cancer vaccines.

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Executive summary

Heat-shock proteins are key players in orchestrating innate & adaptive immunity

- Extracellular heat-shock proteins (HSPs) behave as endogenous danger signals for activation of innate immune responses through binding to Toll-like receptors (TLRs).
- Extracellular HSPs can bind innate ligands and augment innate immune responses through spatiotemporal regulation of chaperoned ligands.
- Extracellular HSP–antigen complexes are cross-presented by antigen-presenting cells via both a TAP-independent endocytic pathway and a TAP-dependent pathway.

HSP release & HSP receptors

- HSPs are released from cells by passive and active mechanisms in response to several kinds of stress.
- TLRs and scavenger receptors have been identified as receptors for HSPs expressed on antigen-presenting cells such as dendritic cells (DCs).

Spatiotemporal regulation of HSPs

- Extracellular Hsp90/ORP150 is targeted to static early endosomes within DCs.
- Extracellular Hsp90–self-DNA/CpG-A complex can stimulate IFN-α production by DCs via spatiotemporal regulation.
- Both extracellular Hsp90 and endogenous Hsp90 may be involved in translocation of exogenous antigen from the endosome to the cytosol for degradation by proteasome, leading to antigen cross-presentation.

Conclusion

- HSPs can link innate and adaptive immune responses, leading to amplified immune responses.
- HSPs are attractive candidates for vaccine development due to their ability to target DCs and to induce specific cytotoxic T cells without the need for an adjuvant.
- Elucidation of the molecular basis for sorting HSPs to static endosomes should lead to the establishment of HSP-based cancer vaccines.

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New Paradigm for Intrinsic Function of Heat Shock Proteins as Endogenous Ligands in Inflammation and Innate Immunity

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New Paradigm for Intrinsic Function of Heat Shock Proteins as Endogenous Ligands in Inflammation and Innate Immunity

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Abstract: Recently, growing evidences that extracellular heat shock protein (HSP) functions as endogenous immunomodulator for innate and adaptive immune responses have been demonstrated. Because HSPs inherently act as chaperones within the cells, passive release such as cell necrosis and active release including secretion in the form of exosome have been suggested for HSP release into extracellular milieu. Such extracellular HSPs have been shown to be activators for innate immune responses through Toll-like receptors (TLRs). However, it has also been suggested that HSPs augmented the ability of associated innate ligands such as LPS to stimulate cytokine production and dendritic cell (DC) maturation. More interestingly, recent study demonstrated that innate immune responses elicited by both endogenous and exogenous danger signals were spatially and temporally regulated and this can be manipulated using Hsp90 or oxygen-regulated protein 150 (ORP150), thereby controlling the immune responses. We will discuss how spatiotemporal regulation of HSP-chaperoned molecules within antigen-presenting cells affects the antigen cross-presentation and innate immune responses. Precise analysis of HSP biology can lead us to establish outstanding HSP-based immunotherapy.

Keywords: Danger signal, dendritic cell, endosome, heat shock protein, innate immunity, Toll-like receptor.

INTRODUCTION

Heat-shock proteins (HSPs), highly conserved across species, are generally considered to be intracellular proteins that have protective functions in situations of cellular stress. A wide variety of stressful stimuli like heat shock, ultraviolet radiation, and viral or bacterial infections induce a substantial increase in intracellular HSP synthesis. The main functions ascribed to HSPs are to act as chaperones of nascent or aberrantly folded proteins. From the immunological point of view, HSPs are of significant interest because HSPs like Hsp70 and gp96 purified from tumor and virus-infected cells are capable of eliciting protective cytotoxic T cell (CTL)-mediated immunity [1, 2]. This immunogenicity is based on antigenic peptides that are associated with Hsp70 and gp96 molecules, and peptide-deprived HSP complexes lose their specific immunogenicity. Recently, there is the growing evidence that the extracellular HSPs play a very important role in the induction of innate immune responses. As HSPs do not have a canonical signal sequence, it has been suggested that HSPs may be released *via* an active secretion mechanism or from cells undergoing necrosis. The resultant extracellular HSPs may then interact with DCs or macrophages, which results in the activation of innate immune responses through TLRs activation. In addition, it has been shown that HSPs can be released in the form of complexes with antigenic peptide and these HSPs-peptide complexes are taken-up by antigen-presenting cells (APCs) such as DCs through specific receptors expressed on APCs. Such peptides may then be transferred to MHC class I molecules through the process known as cross-presentation, and such MHC I-peptide complexes can be recognized by $CD8^+$ T cells leading to T cell activation [2]. Thus, extracellular HSPs act as danger signals for activation of innate immunity as well as enhancers for adaptive immunity of antigen-specific T cell induction. We will discuss the current view of this unique feature of HSPs in the regulation of innate and adaptive immune responses.

IDENTIFICATION OF HSPS-PEPTIDE COMPLEX AS A CANCER ANTIGEN

In their search for tumor-specific antigens, Srivastava's group first identified the ER-resident HSP gp96 as a tumor antigen [2]. Immunization of mice with only μg of gp96 isolated from tumors induced an antitumor immune response through the induction of tumor-specific CTLs. The mechanism for this observed immunity has been shown to be gp96-chaperoned immunogenic antigenic peptides. Following these observations, they further demonstrated that tumor-derived Hsp70 and Hsp90 also bound tumor-specific antigenic peptides. These findings led to the idea that APCs bear HSP-specific receptors on the cell surface. Following the identification of CD91 as a gp96 receptor many receptors have been demonstrated including members of the TLR and scavenger receptor families [3]. When extracellular HSPs are loaded onto APCs, such HSPs can be proinflammatory signals and lead to

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cytokine production through TLR activation as described later. More importantly, HSPs can act as stimulants of adaptive immune responses through their ability to bind antigenic peptides during antigen processing [2, 4-7]. In this case, although the HSP-peptide complexes act as exogenous antigens, they can gain access to the MHC class I Ag presentation pathway, resulting in the stimulation of $CD8^+$ T cell responses, termed cross-presentation. Cross-presentation is required to elicit antitumor $CD8^+$ T cell responses because vaccinated antigenic peptides/proteins act as inherently exogenous antigens. Therefore, how the exogenous Ags such as HSP-peptide complexes get into unusual cross-presentation pathways has been extensively elucidated. Recently, the intracellular pathway for HSP-mediated cross-presentation has been demonstrated. We have shown that an Hsp90-peptide complex was efficiently cross-presented *via* an endosomal pathway but not the classical TAP-dependent pathway [4]. In contrast, protein Ag complexed with Hsp90 is cross-presented by both the endosomal pathway and the proteasome-TAP-dependent pathway [8]. This may be because whole protein is required for further dynamic degradation unlike antigenic peptides.

In vivo, when HSP-peptide complexes are released from dead and dying cells or *via* active secretion from cells, they bind to receptors on APCs and antigens can be delivered to MHC class I molecules on the surfaces of such cells through antigen cross-presentation pathway. Such interactions form the basis for HSP-based anticancer vaccines. The potency of these vaccines has been ascribed to the ability of HSPs to stimulate both innate and adaptive arms of antitumor immune responses through HSP receptors. Therefore, we next overview the HSP receptors expressed on APCs.

HSP RECEPTORS

HSPs interact with a range of receptors expressed on target cells. These receptors are subdivided into 2 groups, one is TLRs and the other is scavenger receptors [9]. The TLRs are major pattern recognition receptors (PRRs) and at least 11 have been identified. At least two TLRs, TLR2 and TLR4, have been demonstrated to function as HSP receptors and can couple the binding of Hsp60, Hsp70, and gp96 to NF-kB activity [10, 11]. In addition, the cell-surface protein CD14, which couples LPS exposure to TLR4 activation, is also required for Hsp70-mediated induction of cytokines TNF-α, IL-1β, and IL-6 [12]. In a series of studies by Lehner, immunological consequences of Hsp72 can be localized to specific domains of the Hsp72 molecule. C-terminal portion of Hsp72 (aa 359-610) stimulates production of chemokines, IL-12, TNF-α, and NO, induces T helper cell type 1 polalization, and stimulates the maturarion of DCs. The N-terminal ATPase domain (aa 1-358) largely lacks these functions [13]. Wheeler *et al.* have also demonstrated that C-terminus region of Hsp72 served as an activator for macrophages to produce TNF-α. These effects did not seem to result from LPS contamination [14].

However, some studies suggested that these interactions between Hsp70 and TLR are not likely to be exerted through the direct binding of Hsp70 to CD14, TLR2, or TLR4, as cells stably transfected with CD14, TLR2, or TLR4 do not bind avidly to Hsp70 [15]. These data suggest that low-affinity interactions may be involved in TLR activation by Hsp70. Previous study have shown that TLR activation by Hsp60 requires the internalization of the Hsp60; therefore, experiments using cells with simple TLR gene overexpression may thus be inadequate to assess direct HSP-TLR binding [16].

Scavenger receptors (SRs) constitute the other family of PRRs. The SRs are receptors for chemically modified forms of lipoproteins, including oxidized and acetylated low-density lipoproteins. The SR family is subdivided into eight different subclasses (A to H) and many receptors belonging to this family are expressed on the antigen-presenting cell surface. The oxidized LDL-binding protein CD91/LRP has been shown to be a common receptor for Hsp60, Hsp70, Gp96, and calreticulin [17]. However, Theriault showed that the difference of Hsp70 binding to CD91-positive cells and negative cells was minimal. Therefore, the Hsp70 binding to CD91 may be a low-affinity interaction or be indirect. It has been demonstrated that Hsp70 can interact with at least three members of the SR family, LOX-1 [18], SREC-1 [19], and FEEL-1 [19]. Hsp70 can be bound at high affinity by these SRs and internalized [20]. Both Hsp90 and Hsp60 can also bind to LOX-1. In addition, gp96 and calreticulin show significant affinity to SR-A1 and SREC-1 and are internalized by these receptors [21, 22]. However, HSP (gp96) binding to SR-A1 is immunosuppressive [23], whereas LOX-1 mediates Hsp70 immunogenicity and antigen presentation [18]. Therefore, detailed studies of each receptor subtype and its interaction with others are essential to determine the effects on immune responses of cellular interactions with HSPs.

MECHANISM OF HSP RELEASE FROM CELLS

As described above, immunization with HSP-peptide complexes elicits antitumor immune responses *via* cross-presentation by APCs. Do immune responses induced by extracellular HSP-mediated Ag cross-presentation actually take place *in vivo*? Because HSPs are inherently intracellular proteins, some mechanism for the release of endogenous HSPs into extracellular space must exist. However, HSPs are not secreted *via* the classical pathway because their sequences encode no secretion leader signals. In fact, as described later, it has been shown that the export of HSP to extracellular space could not be blocked by typical inhibitors of ER-Golgi pathway, such as brefeldin A. Currently, two mechanisms are considered to result in release of HSPs from cells; passive release mechanisms, including necrotic cell death, severe trauma, surgery and following infection with lytic

viruses, and active release mechanisms involving nonclassical protein release pathways.

Passive Release Mechanism

Release of intracellular proteins involves cell lysis and this may occur in pathological conditions that give rise to necrosis. Basu and coworkers reported that HSPs, including gp96, Hsp90, Hsp72 and calreticulin, are released from cells by necrotic, but not apoptotic, cell death [17]. Necrotic cell death results in the release of intracellular contents into the extracellular space, thereby liberating HSPs. These results make the cell necrosis an attractive explanation for the mechanism by which HSPs are released into the extracellular milieu as an intrinsic immunological messenger.

Active Release Mechanism

An active release mechanism has been suggested as an additional mechanism to the passive release hypothesis. Asea *et al.* showed that IFN-γ and IL-10 induce the active release of constitutively expressed Hsp70 (Hsc73) as well as Hsp72 from tumors under conditions that will not induce cell death [24]. Because IFN-γ and IL-10, but not the anti-inflammatory cytokine TGF-β, is considered to have high concentrations within inflammatory foci, these proinflammatory cytokines may mediate the active release of Hsc73 and Hsp72 [25]. Moreover, Asea's group showed that whereas some extracellular Hsp72 could be found as free Hsp72, a proportion of extracellular Hsp72 was released within exosomes [25, 26]. Exosomes are internal vesicles of multivesicular bodies (MVB) released into the extracelluar milieu upon fusion of MVB with the cell surface. Additionally, they showed that Hsp72 was released by a nonclassical protein transport pathway and that intact surface membrane lipid rafts were required for efficient stress-induced Hsp72 release. Mambula *et al.* also demonstrated that a prostatic cancer cell line secreted Hsp72 *via* an endolysosomal pathway [27]. Furthermore, Fleshner *et al.* demonstrated another mechanism for the Hsp72 release under condition of psychological stress [28]. They proposed that activation of the sympathetic nervous system by stimuli, such as stressor exposure results in the release of norepinephrine and the subsequent activation of α1-adrenergic receptors (ADRs). Stimulation of α1-ADRs results in an increase in intracellular Ca^{2+}, which may stimulate the release of exosomes containing Hsp72. More recently, Vega *et al.* have demonstrated that Hsp72 was found inserted into the plasma membrane of cells after stress, which may be derived by inverse evagination [29, 30]. In addition, Hsp72 was released into the extracellular spaces in a membrane-associated form (i.e. exosome) of which could act as a danger signal to activate the macrophages. Strikingly, activation of macrophages by membrane-associated form of Hsp72 was 260-fold more effective than the free recombinant Hsp72. This robust effect is likely due to the high concentration of Hsp72 within the vesicle (exosome).

Taken together, these studies suggest that the active release hypothesis is an important mechanism by which Hsp72 is released into the circulation. However, it remains to be conclusively demonstrated that T cell responses are primed by active release of Hsp72, especially in the case of psychological stress as well as tumor bearing host' immune surveillance through the cross-presentation of a tumor-derived extracelluar HSP-peptide complex.

DO HSPS ACT AS ENDOGENOUS DANGER SIGNALS?---THAT IS THE QUESTION!

From the Point of View that the HSPs Act as Chaperokines

Preparations of HSPs such as Hsp60, Hsp70, Hsp90 and gp96 purified from a variety of sources including bacteria and mammalians, as well as recombinant bacterial and human products, have been reported to be potent activators of innate immunity. Specifically, these HSP preparations have been reported to stimulate the production of proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-12 and the release of NO and C-C chemokines by monocytes, macrophages, and DCs [31-34]. HSPs are also reported to induce the maturation of DCs, as demonstrated by the up-regulation of MHC class I and II molecules and costimulatory molecules such as CD80, CD86, and CD40 [17]. Thus, it has been proposed that, through their cytokine-like functions, HSPs may serve as endogenous "danger signals" to the host immune system at sites of tissue injury or stress where HSPs are released into extracellular spaces. The discovery of the extracellular biological actions of HSPs as "chaperokines" is very attractive and the chaperokine theory is interesting with regard to the extracellular HSPs as a cause of sterile inflammation.

Microbial Contamination may be Responsible for Immune Activation

In contrast, there have been many criticisms stating that these chaperokine activities were due to microbial contamination. The representative microbial products, LPS is a strong stimulus of innate immune signaling. Because microbial contamination occurs during purification of HSPs, and the recombinant HSPs expressed within *E. coli* inherently are contaminated with LPS, the observed biological actions might be artifact due to microbial products. To examine this possibility, using highly purified HSP preparations, a series of experiments was performed and demonstrated that the HSP cytokine functions are in fact a result of contaminating bacterial products. Wallin *et al.* noted that highly purified murine liver Hsp70 had no cytokine effects, even at concentrations as high as 200-300 mg/ml [35]. In contrast, an LPS-contaminated preparation at Hsp70 concentrations as low as 0.05-0.1 mg/ml induced cytokine production that was heat-sensitive and not inhibitable by polymixin B. Bausinger *et al.* demonstrated that recombinant human Hsp70, after further purification to remove LPS contamination, was no longer capable of inducing the activation of

DCs [36]. Furthermore, Gao *et al.* reported that the cytokine-inducing activity of LPS was heat-sensitive and that the ability of commercially available recombinant human Hsp70 to induce TNF-α production in macrophages was entirely a result of contaminating LPS, and that of recombinant human Hsp60 was a result of contamination by LPS as well as LPS-associated molecules [37, 38]. These investigators demonstrated further that highly purified Hsp60, Hsp70, and gp96, which were essentially free of LPS contamination, retained their normal biological properties, including the molecular chaperone function and ATPase activity. Thus, Hsp60, Hsp70, Hsp90, and gp96 do not have cytokine-inducing activities as reported previously. Moreover, Gao *et al.* using a gene expression array, showed that Hsp60 and Hsp70 had no effect on the expression of 96 common cytokine genes in macrophages [39].

HSPs Augment the Actions of Associated Microbial Products

However, some HSPs have been shown to bind microbial products very efficiently and thus augment the biological actions of microbial products. Habich *et al.* have demonstrated that Hsp60 bound LPS tightly and that Hsp60-bound LPS, but not Hsp60 itself, was responsible for the observed cytokine effects of Hsp60 preparation [40]. More interestingly, Hsp60-bound LPS was more potent than LPS in inducing cytokine production. Reeds *et al.* have shown that gp96 binds endotoxin in a high affinity, saturable and specific manner. The same study demonstrated that low (< 0.27 EU endotoxin/mg protein) endotoxin preparations of gp96 do not stimulate NF-κB activation or NO release in macrophages [41]. In addition, Wager *et al.* showed that gp96 bound lipid-based TLR ligands such as palmitoyl-3-Cys-Ser-$(Lys)_4$ (Pam_3Cys; a TLR2 ligand) and LPS (TLR4 ligand). Binding of Pam_3Cys and LPS to gp96 enhanced their ability to induce the activation and maturation of DCs [42]. Thus, there may be cooperation between gp96 and TLR ligands and that the former amplify the effects of the latter. These results suggested that very low concentrations of LPS if "chaperoned by a certain HSP" could induce cytokine production. In this context, HSP augments immune responses against molecules chaperoned by HSPs, but not against HSPs themselves. In the clinical setting, this unique character of HSPs may contribute to the *in vivo* recognition of Gram-negative bacterial infection by binding to LPS and augmenting the host defense system *via* TLR4. Although further studies are necessary to determine whether the reported cytokine-producing effect is a result of HSPs or contamination of TLR ligands, there is no doubt that HSPs play very important roles in eliciting immune responses by acting as danger signals *via* HSPs themselves and/or HSP-associated molecules.

"Microbial Product-Free" Cell Surface-Expressed HSPs Elicit Immune Responses

To avoid the problem of microbial contamination, a number of studies involving transgenic expression of HSPs on the cell surface have been conducted. It has been demonstrated that cell-surface HSPs can activate immune responses. Under these conditions, contamination by exogenous molecules such as LPS is unlikely. Zheng *et al.* demonstrated that cell surface expression of gp96 on tumor cells induced efficient T cell priming and tumor rejection *in vivo* [43]. Likewise, Chen *et al.* showed that transgenic expression of Hsp70 on the tumor cell surface elicited antitumor immunity, and immunization of mice with these tumor cells led to induction of tumor-specific cytotoxic T cells [33]. In addition, the transgenic expression of gp96 on the cell surface leads to *in vivo* activation of DCs and the development of lupus-like systemic autoimmune disease in mice [44, 45]. Vaccination of mice with gp96-secreting fibroblasts leads to *in vivo* activation of $CD11b^+$ and $CD11c^+$ APCs [46]. These studies have demonstrated that cell-surface HSPs are capable of activating the immune system *in vivo*. However, it remains unclear whether the observed *in vivo* effects are caused by the direct effects of HSPs themselves or *via* HSP-associated molecules.

To Further Expand the Chaperokine World

In this field, it is very important that HSP purification procedures should be carried out to the highest standards of molecular chaperone purification as described by Pockley *et al.* [47]. In addition, identification of receptor binding domain of each HSP should be identified. More importantly, X-ray crystallographic analysis may reveal the mode of HSP-receptor binding. From the biological point of view, as endogenous danger signal, under the bacterial and viral infection, damaged cell-derived extracellular HSPs may bind microbial products such as LPS, lipoproteins, bacterial DNA and viral RNA in the inflamed milieu, leading to activation of TLR-, and non-TLR-mediated innate immune responses. This nascent but very attractive field of biology should be further developed through careful experiments.

ENDOGENOUS DANGER SIGNALS AND STERILE INFLAMMATION CONCOMITANT WITH IMMUNE RESPONSES

In addition to recognizing pathogen-associated molecular patterns (PAMPs), the immune system has evolved to recognize endogenous danger signals, called damage-associated molecular patterns (DAMPs), many of which are released by dying or necrotic cells and contribute to "sterile inflammation" in a noninfectious sterile setting. Furthermore, the recognition of DAMPs can activate the innate immune system *in vivo*. DAMPs are structurally and sequence-diverse family of endogenous molecules, generally intracellular and hidden by the plasma membrane, often released from necrotic cells. To date, it has been demonstrated that uric acid [48], DNA and more specifically unmethylated CpG-rich DNA regions, HMGB1 [49, 50], SAP130 [51], IL-1α [52], IL-33 [53, 54], S100 proteins [55], and HSPs act as DAMPs [56]. However, recent study has suggested that highly purified HMGB1 does not induce significant amounts of