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Heat immunotherapy with heat shock protein expression by hyperthermia using magnetite nanoparticles

Akira Ito[1], Takeshi Kobayashi[2], and Hiroyuki Honda[3]

[1] *Department of Chemical Engineering, Faculty of Engineering, Kyushu University,*
[2] *Department of Biological Chemistry, School of Bioscience and Biotechnology, Chubu University,*
[3] *Department of Biotechnology, School of Engineering, Nagoya University*

Abbreviations: heat shock protein, HSP; drug delivery system, DDS; magnetite cationic liposome, MCLs; antibody-conjugated magnetoliposome, AML; monoclonal antibody, MAb; natural killer, NK; metallothionein-I, MT; glucose-regulated protein 96, gp96; major histocompatibility complex, MHC; endoplasmic reticulum, ER; transporter associated with antigen processing, TAP; cytotoxic T lymphocyte, CTL; antigen-presenting cell, APC; dendritic cell, DC; nuclear factor, NF; tumor necrosis factor, TNF; interleukin, IL; macrophage inflammatory protein, MIP; macrophage chemotactic protein, MCP; regulated upon activation normal T cell expressed and secreted, RANTES; nitric oxide, NO; granulocyte macrophage-colony stimulating factor, GM-CSF; interferon, IFN.

Abstract

Hyperthermia is a possible approach for cancer therapy. However, a major technical problem associated with the use of hyperthermia is the difficulty of heating the local tumor region to the intended temperature without damaging normal tissue. Accordingly, in hyperthermia treatment, the expression of heat shock proteins (HSPs) has been considered a complicating factor because the expression of HSPs protects cells from apoptotic cell death. In cancer immunity, on the other hand, HSPs, including HSP70, have been shown to play an important role in immune reactions. If HSP expression induced by hyperthermia is involved in tumor immunity, novel cancer immunotherapy based on hyperthermia treatment can be developed. In such a strategy, a tumor-specific hyperthermia system that can induce necrotic cell death via HSP expression without damaging non-cancerous tissues would be highly advantageous. An intracellular hyperthermia system using functionalized magnetite nanoparticles, including magnetite cationic liposomes and antibody-conjugated magnetoliposomes, facilitates tumor-specific hyperthermia; this can induce necrotic cell death via HSP expression, which in turn induces antitumor immunity. We term this novel cancer therapy as "heat immunotherapy." This review discusses recent progress in cancer immunology via HSP expression and novel immunotherapy based on hyperthermia.

Keywords: Heat shock proteins, Hyperthermia, Tumor immunity, Magnetite

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Introduction

Hyperthermia is a possible approach for cancer therapy. However, a major technical problem associated with the use of hyperthermia is the difficulty of heating the local tumor region to the intended temperature without damaging normal tissue. The rationale underlying hyperthermia is based on the fact that temperatures over 42.5°C are cytotoxic to tumor cells[1], particularly in an environment with a low pO_2 and low pH, conditions that are typically found in tumor tissue due to insufficient blood perfusion. At higher temperatures (e.g., 45–46°C), a greater number of tumor cells can be killed and, in principle, tumor-specific hyperthermia can kill all kinds of tumor cells because hyperthermia is a physical treatment and therefore would have fewer side effects than chemotherapy or radiotherapy.

Using magnetite nanoparticles, we have developed an intracellular hyperthermia system that can control local temperature within tumors. This novel hyperthermia treatment has produced unexpected biological responses, including overcoming thermotolerance due to specific heating of the tumor at high temperature, and an antitumor immune response induced by the expression of heat shock proteins (HSPs). These results suggest that our hyperthermia system can kill not only heated tumors but also non-heated tumors, including metastatic cancer cells. We have investigated the role of HSP70, which is a well-defined HSP, in order to elucidate the mechanism of immune induction by hyperthermia. In the present ar-

Correspondence to: Akira Ito, Ph.D., Associate Professor of Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan. Tel: 81-92-802-2753, Fax: 81-92-802-2793, E-mail: akira@chem-eng.kyushu-u.ac.jp

ticle, the mechanism of the anticancer immune response induced by hyperthermia and new cancer immunotherapy based on the mechanism are reviewed and discussed.

Hyperthermia using magnetite nanoparticles

Various heating methods have been applied in hyperthermia[2]. However, an inevitable technical problem with hyperthermia is the difficulty of uniformly heating only the tumor region to the required temperature without damaging normal tissue. Accordingly, some researchers have proposed the use of "intracellular" hyperthermia to achieve a tumor-specific hyperthermia system, and have developed submicron magnetic particles (typically less than 100 nm in diameter) for this purpose.

In 1979, Gordon *et al.* first proposed the concept of intracellular hyperthermia. Magnetic iron oxide (Fe_3O_4, magnetite) particles of 6 nm diameter suspended in a sucrose solution were injected into a tail vain, and the selective uptake of particles by the tumor was observed by light and electron microscopy[3]. In 1999, Jordan *et al.* developed aminosilan-coated magnetite nanoparticles, in order to enhance the cellular uptake of the particles[4], and they demonstrated that intracellular hyperthermia exhibited greater cytotoxicity compared to water bath heating. When magnetite nanoparticles are used in *in vivo* study, the lack of colloid stability is an important issue. A possible approach to overcome these shortcomings of magnetic colloids is to coat magnetite nanoparticles with dextran; dextran magnetite was thus developed[5-7].

For drug delivery systems (DDS), liposomal coating is a promising approach. Shinkai *et al.* developed DDS techniques using liposomes for intracellular hyperthermia (Fig. 1A). The accumulation of magnetite nanoparticles in tumor cells can be enhanced by conferring a positive surface charge to the liposomal surface, and these authors have developed "magnetite cationic liposomes (MCLs)" with improved adsorption and accumulation properties[8]. MCLs, which have a positive surface charge, have 10-fold higher affinity for T-9 rat glioma cells than neutrally charged magnetoliposomes. Furthermore, a significant development in intracellular hyperthermia occurred when Shinkai and colleagues developed antibody-conjugated liposomes containing magnetite nanoparticles (antibody-conjugated magnetoliposomes, AMLs). They constructed immunoliposomes using mouse G22 monoclonal antibody (MAb) against human glioma cell lines[9], mouse G250 MAb against a human renal cell carcinoma cell line[10], and humanized MAb against human epidermal growth factor receptor-2 (Herceptin^R)[11], and demonstrated the tumor-specific targeting ability of these AMLs.

The first *in vivo* intracellular hyperthermia experiment was performed by Gordon *et al.* in 1979[3], using rats bearing mammary tumors. Non-coated magnetite nanoparticles were injected into a vein. Microscopic tissue analysis revealed that tumor cells had taken up particles. Two days after injection, the rats were exposed to a 450-kHz alternating magnetic field for 12 min. The tumor temperature increased by 8°C and histologic evidence of tumor necrosis was demonstrated. It was also reported that there were no side effects or toxicities associated with the use of the particles. Since the first *in vivo* experiment by Gordon, many researchers have reported encouraging results from intracellular hyperthermia using magnetite nanoparticles, including dextran magnetite[6,12] and aminosilane-coated magnetite[12,13]. One of the most systematic and comprehensive experiment using liposomal magnetite has been conducted by us (Fig. 1B). We have demonstrated the efficacy of intracellular hyperthermia using magnetite nanoparticles covered with liposomes in animals with several types of tumor, including B16 mouse melanoma[14], MM46 mouse mammary carcinoma[15], PC3 and LNCaP human prostate cancer in athymic mice[16], spontaneously developed primary melanoma in transgenic mice[17], T-9 rat glioma[18], Os515 hamster osteosarcoma[19], and VX-7 squamous cell carcinoma in rabbit tongue[20]. In these therapeutic experiments, MCLs were directly injected into solid tumors and the animals were irradiated several times (repeated hyperthermia) for 30 min with an alternating magnetic field of 118 kHz. The temperature of the tumor was elevated rapidly by magnetic heating and reached the intended temperature (42–46°C). In contrast, the rectal temperature or that in a tumor lacking the MCLs did not increase. After the alternating magnetic field irradiation, the tumor volume decreased markedly, and complete tumor regression was observed in 96% (46/48) of the animals in these experiments. These results indicate that MCLs are the superior tool for hyperthermia and that repeated hyperthermia using magnetite nanoparticles is a promising approach for cancer therapy.

We observed an antitumor immune response induced by hyperthermia using MCLs in an experimental T-9 rat glioma model in which a tumor was transplanted into each femur of a rat[21]. Interestingly, although only one tumor was subjected to hyperthermia, the other tumor also disappeared completely. Immunohistochemical assay revealed that natural killer (NK) cells and CD8- and CD4-positive T cells had migrated into the tumors after the hyperthermia treatment[21]. Here, T-9 rat glioma is a highly immunogenic cell line. Accordingly, our protocol was applied to a hereditary melanoma model; a primary skin melanoma developing in a metallothionein-I (MT)/*ret* transgenic mouse line[17]. In that study, in order to investigate whether an immune response could be induced after hyperthermia in MT/*ret* transgenic mice, the sizes of all the tumors that spontaneously occurred throughout the mouse body were measured. Each mouse (control group and hyperthermia group) had 8 tumors with sizes

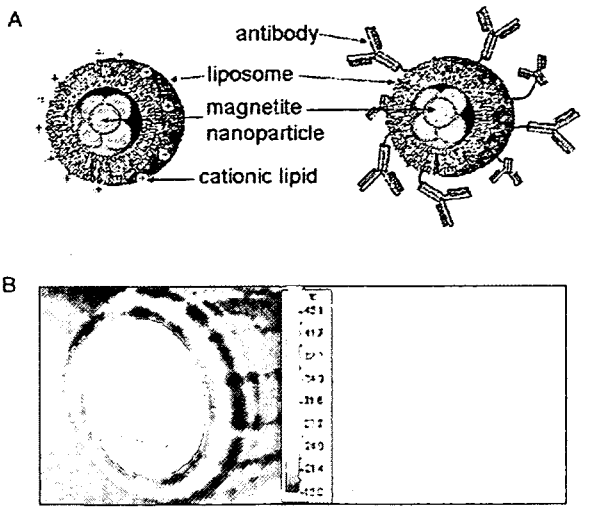

Fig. 1 Hyperthermia using magnetite nanoparticles.
(A) Liposomal drugs containing magnetite nanoparticles for drug delivery of magnetite nanoparticles. Left, magnetite cationic liposome (MCL); right, antibody-conjugated magnetoliposome (AML).
(B) Thermography of a mouse during hyperthermia using MCLs. MCLs were injected into the subcutaneous B16 tumors of mice and then an alternating magnetic field was irradiated. An alternating magnetic field was generated by a horizontal coil with a transistor inverter. The mouse was placed inside the coil. The left and right pictures are photographs of the mouse taken during and immediately after the alternating magnetic field irradiation, respectively. These pictures demonstrate that the MCL-mediated hyperthermia system is able to heat the tumor specifically.

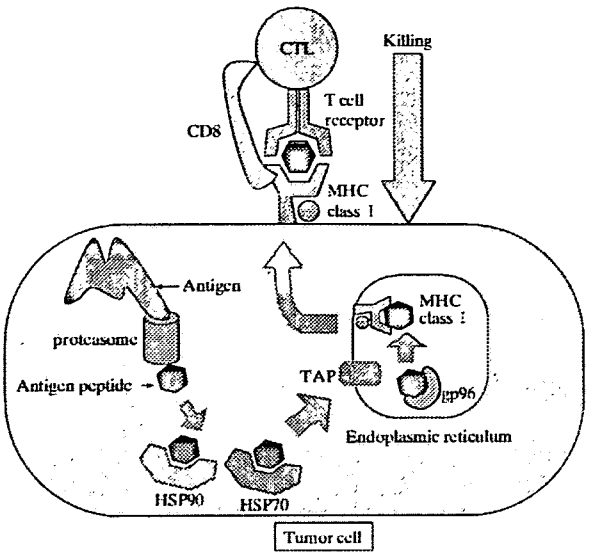

Fig. 2 Relay line model for tumor antigenic peptide transfer during antigen processing and presentation by heat shock proteins (HSPs). The HSP family, including HSP70 and HSP90 in the cytoplasm, and gp96 in the endoplasmic reticulum (ER), is involved in peptide transfer to MHC class I molecules. Details are described in the text.

ranging from 2 to 8 mm, which were located in the back and tail. In the control mice, all tumors grew and no tumor regression was observed. In the hyperthermia group on the other hand, although only 1 tumor was treated twice with hyperthermia, the other 3 tumors (2 on the back and 1 on the tail) also regressed and disappeared.

A mechanism of antitumor immunity induced by hyperthermia

HSPs were originally described with respect to their roles as chaperones induced by temperature shock as well as various other types of stress[22]. Recently, an additional role has been ascribed to HSPs as activators of the immune system. The importance of the HSP family, including HSP70, HSP90, and glucose-regulated protein 96 (gp96), in immune reactions has been demonstrated[23-26], and several researchers have reported that heat-treated tumor cells can play a vaccine-like role and elicit antitumor immunity[27-29]. With regard to the mechanism of antitumor immunity induced by hyperthermia using MCLs, our findings suggest 2 possible mechanisms of antigen presentation via HSP70 expression during hyperthermia[30-32]. One possible mechanism is the heat-induced augmentation of tumor immunogenicity due to the presentation of antigenic peptides via the major histocompatibility complex (MHC) class I antigens of tumor cells. Srivastava *et al.* proposed the "relay line model"[33-35] for tumor antigenic peptide transfer during antigen processing and presentation by HSPs (Fig. 2): The peptides digested by the proteasome are first bound to HSP70 or HSP90, which carry them to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP); the peptides are then transferred to gp96 in the lumen of the ER; and in the terminal step, gp96 transfers the peptides to the MHC class I $-\beta_2$ microglobulin complexes, and the tumor cell presenting the peptide via MHC class I is killed by MHC class I-restricted cytotoxic T lymphocytes (CTLs). Wells *et al.* demonstrated that stably transfected B16 melanoma cells that constitutively expressed human HSP70 exhibited significantly increased levels of MHC class I antigens on their surface[36,37]. We have demonstrated that augmentation of MHC class I antigens on the tumor cell surface via HSP70 expression causes immune induction[30]. In that study, HSP70 expression reached a maximum 24 h after heating, and the augmentation of MHC class I surface expression began 24 h after heating and reached a maximum 48 h after heating. In an *in vivo* experiment, the growth of T-9 cells in immunocompetent syngeneic rats was significantly inhibited by hyperthermia, with augmentation of MHC class I antigen surface

expression. Conversely, the growth of T-9 cells in nude rats was not inhibited, suggesting that the effector cells were T lymphocytes. Furthermore, compared with lymphocytes from non-immunized rats or rats injected with non-heated T-9 cells, the splenic lymphocytes of rats injected with heated T-9 cells displayed specific cytotoxicity against T-9 cells. These results suggest that HSP70 is an important modulator of tumor cell immunogenicity during hyperthermia.

An alternative mechanism of recognition of tumor cell antigens by the host immune system in hyperthermia is the cross-presentation of antigenic peptides by dedicated antigen-presenting cells (APCs). HSP-mediated antitumor immunity may be caused by a vaccine-like effect of HSP-peptide complexes released from dying tumor cells. The released HSP-peptide complexes encounter APCs that express receptors such as CD91[38], CD40[39], and Toll-like receptors 2 and 4[40]. The interaction of HSP-peptide complexes with these receptors leads to receptor-mediated endocytosis, processing of the antigenic peptide by the endogenous MHC class I pathway, and re-presentation on the cell surface to CD8-positive T cell receptors[41]. Additionally, HSP70 functions as a direct activator of APCs, including dendritic cells (DCs)[42]. When HSPs bind to signaling receptors, such as CD14[42] and/or CD91 receptors[38], that trigger nuclear factor (NF)-$\kappa\beta$ activation[43], the DCs release pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-12[42, 44]; chemokines, including macrophage inflammatory protein (MIP)-1, macrophage chemotactic protein (MCP)-1, and regulated upon activation normal T cell expressed and secreted (RANTES)[45]; and nitric oxide (NO)[46], and up-regulate the expression of costimulatory and MHC class II molecules[43, 47, 48], resulting in the maturation of DCs[36]. This cytokine-like ability of HSP70 to stimulate the innate immune system is independent of the peptides they chaperone, suggesting that HSP70 is a natural adjuvant. Classical immunology theory is based on the distinction between "self and non-self"; however, this self-nonself model is sometimes not very useful, particularly in cancer immunity. Matzinger proposed the "Danger model," which suggests that the immune system is more concerned with damage than with foreignness[49, 50]. In the "Danger model," HSPs are considered an important "Danger signal." In particular, when HSPs, which are normally present in the cytoplasm, are released into the bloodstream, they can act as a signal to the immune system indicating an abnormal situation. We demonstrated that HSP70 expression following hyperthermia using MCLs induced antitumor immunity in rats with T-9 rat glioma[31]. Because MCLs are themselves heated in our hyperthermia system, the distribution of magnetite nanoparticles within tumors is an important issue. When MCLs were repeatedly heated, the surrounding tumor tissues underwent necrosis, and magnetite nanoparticles subsequently expanded into the necrotic area within the tumor, resulting in wide distribution of magnetite nanoparticles. Thus, the entire tumor area was necrosed by repeated (3 times) hyperthermia (with a 24-h interval over 3 days). The 24-h interval corresponded to the time when HSP70 expression in the T-9 cells reached its maximum, and a large amount of HSP70 was detected in the tumor tissue. Thus, our hyperthermia system using MCLs overcame thermotolerance and induced necrotic cell death that correlated with HSP70 expression. Next, we purified the HSP70-peptide complexes from the tumor after hyperthermia, and found that immunization of rats with T-9-derived HSP70 strongly suppressed tumor growth. HSP70-peptide complexes from liver (control) were also purified, and their vaccine effects were examined; however, no antitumor effects were observed. These results suggest that HSP70 in tumor cells chaperones some antigenic peptides after hyperthermia.

Although our hyperthermia system is designed to induce necrotic cell death, in order to induce HSP expression, there has been controversy in cancer immunology over whether vaccination with apoptotic cells or necrotic cells is more efficient. Apoptosis is programmed cell death, and is also considered "clean" cell death because the contents of the cells (including tumor antigens) are not released into the external environment but become packaged into the apoptotic body. In contrast, necrotic cell death is considered an unprogrammed event that is "not clean" because the cell contents are released into the environment. In necrotic cell death, as mentioned above, APCs including DCs can recognize antigens released from tumor cells via HSPs[43, 51]. In apoptosis, apoptotic bodies are engulfed by APCs, followed by antigen processing and presentation of tumor antigens by MHC class I antigens[52]. In fact, appropriate processing by APCs may occur in both apoptosis and necrosis. Apoptotic cell death would appear to be preferable to necrotic cell death because of the predictability of the results of apoptosis. When the complex program of apoptotic cell death is fully decoded, it will be possible to control the activation of a specific cascade of apoptotic events and induce apoptosis in any type of cancer using techniques such as molecular target therapy. However, at present, cancer therapy specifically designed to induce apoptosis do not appear to be feasible because cancer cells are adapted to escape from "death," particularly from apoptotic cell death, due to continual gene mutation. In contrast, it is relatively easy to induce necrotic cell death. Generally, when cells undergo extreme stress, they die in a necrotic manner. Yonezawa *et al.* examined the manner of cell death induced by hyperthermia[53]. Apoptotic cell death was induced in malignant fibrous histiocytoma cells by mild hyperthermia treatment at 42°C, whereas necrotic cell death was induced by hyperthermia at 44°C. Thus,

hyperthermia can easily induce necrotic cell death (in principle, in any type of tumor cell) by heating cells to a sufficiently high temperature. Our intracellular hyperthermia treatment can heat the tumor specifically via the MCLs. Moreover, the amount of heat generated in the tumor can be controlled by modulating the magnetic field intensity, making it possible to induce necrotic cell death without damage to the surrounding normal tissues. Another reason why necrotic cell death is effective for cancer therapy is that necrosis may strongly induce a "Danger signal." We observed that numerous and diverse kinds of immunocytes, such as CD8- and CD4-positive T-cells, NK cells, macrophages, and DCs, infiltrated into the necrotic area of tumors after hyperthermia treatment[21,31]. Todryk *et al.* observed infiltration of such cells into B16 melanoma nodules transfected with the HSP70 gene[54], suggesting that HSP70 expression is a "Danger signal" for the recruitment of immunocytes.

Taken together, the "passive release" of HSPs via heat-controlled necrosis by hyperthermia using MCLs is clearly an important methodology. Recently, on the other hand, an additional mechanism for HSP release has been proposed as "active release" [55]. Lancaster and Febbraio demonstrated that exosomes provide the major pathway for secretory vesicular release of the inducible type of HSP70[56]. In addition to containing HSP70, exosomes are densely packed with immunostimulatory mediators, including MHC class I and II[57] and costimulatory molecules[58]. Interestingly, psychological stress induced a marked increase in circulating HSP70; psychological stress induced by exposure of a rat to a cat resulted in the release of inducible type of HSP70[59]. Further studies remain to investigate whether hyperthermia treatment facilitates the active release of HSPs.

A proposed scenario in which HSPs function during successive stages of an antitumor response after hyperthermia is summarized and illustrated in Fig. 3.

Development of novel cancer immunotherapy based on heat-induced immune response via HSP expression

We are developing novel cancer immunotherapy based on the mechanism of anticancer immune response via HSP expression. Three key elements may be involved in the mechanism based on heat-induced immune response: (i) CTL as an effector cell, (ii) APC as an antigen-processing and antigen-presenting cell for HSP-peptide complex released from necrotic cells, and (iii) HSPs as natural and powerful immunostimulants.

Immunotherapy using cytokines has become an accepted therapeutic modality. We undertook these studies in order to study the combined effects of cytokines and hyperthermia using MCLs on melanoma[60]. MCLs were injected into a B16 melanoma nodule in mice, which were subjected to an alternating magnetic field for 30 min. The temperature at the tumor reached 43°C and was maintained by controlling the magnetic field intensity. At 24 h after hyperthermia, IL-2 or granulocyte macrophage-colony stimulating factor (GM-CSF) was injected directly into the melanoma. Mice were divided into 6 groups: group I (control), group II (hyperthermia), group III (IL-2), group IV (GM-CSF), group V (hyperthermia + IL-2), and group VI (hyperthermia + GM-CSF). Complete regression of tumors was observed in the mice of groups V and VI [75% (6/8) and 40% (4/10) of the mice, respectively], while no tumor regression was observed in mice of the other groups.

In addition, we conducted a screening of cytokines to enhance the vaccine effects of heated tumor cell lysate[61]. After heating mouse melanoma B16 cells (43°C, 30 min) to elicit increased HSP70 expression, the cells were lysed by freeze-thawing in order to prepare heat-treated cell vaccine. In approaches using poorly immunogenic melanoma B16, various cytokines (IL-1β, IL-2, IL-4, IL-6, and IL-12, interferon [IFN]-β and -γ, GM-CSF, and TNF-α) were assessed for combination with heat-treated cell vaccine. Syngeneic mice were immunized subcutaneously using heat-treated cell vaccine twice, on days –14 and –7, while cytokines were injected intraperitoneally on day –7. Subcutaneous B16 cell challenge was performed on day 0. IL-12 appeared to significantly enhance the vaccine effects of heat-treated cell vaccine, compared to non-heated cell lysate vaccine and non-vaccination. Systemic administration of recombinant IL-12 could augment the efficacy of heat-treated cell vaccine, inducing protective immunity against tumor challenge and enhance systemic cytotoxic activity in splenocytes against B16 cells in treated mice.

DCs are potent antigen-presenting cells that play a pivotal role in regulating immune responses in cancer and have recently been demonstrated to be activated by HSPs. For the use of APCs as antigen-processing and antigen-presenting cells for HSP-peptide complexes released from necrotic cells, DC therapy was combined with hyperthermia using MCLs[62,63]. In an *in vitro* study[62], when immature DCs were pulsed with mouse B16 cells heated at 43°C, MHC class I and II and costimulatory molecules CD80 and CD86 in the DCs were up-regulated, thus resulting in DC maturation. Mice bearing a melanoma nodule were subjected to combination therapy using hyperthermia and DC immunotherapy *in vivo* by means of hyperthermia using MCLs and directly injected immature DCs. The mice were divided into 4 groups: group I (control), group II (hyperthermia), group III (DC therapy), and group IV (hyperthermia + DC therapy). Complete regression of tumors was observed in 60% of the mice in group IV, while no tumor regression was observed among the mice in the other groups. Increased CTL and natural killer (NK) activity was ob-

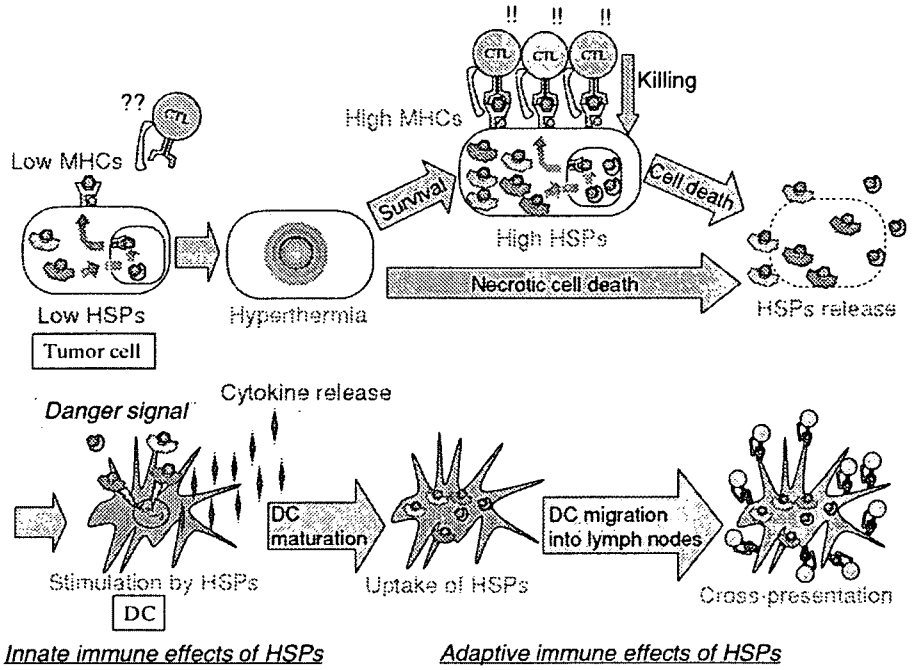

Fig. 3 Proposed scenario for the mechanism of anticancer immune induction by hyperthermia. Tumor cell with a low concentration of intracellular heat shock protein (HSP)-peptide complexes, decreased function of the endogenous antigen processing machinery, and a very low level of MHC class I-peptide complexes at the cell surface. Cytotoxic T lymphocytes (CTLs) are unable to locate tumor cells because of the low level of MHC class I expression, which causes poor immunogenicity of tumor cells. Hyperthermia treatment results in increased levels of intracellular HSP-peptide complexes, enhanced processing of endogenous antigens, and an increase in the density of MHC class I-peptide complexes at the cell surface. These tumor cells are then recognized directly by CTLs. Dying tumor cells, which are killed by the CTLs or by lethal hyperthermia treatment, release their intracellular contents, including HSP-peptide complexes. The released HSPs, acting as a "Danger signal," activate neighboring monocytes to produce proinflammatory cytokines and recruit antigen-presenting cells (APCs), including dendritic cells (DCs). The HSP-peptide complexes are taken up by DCs. Then DCs migrate into lymph nodes and are in turn presented to T cells via MHC class I and/or II antigens (cross-presentation).

served in an *in vitro* cytotoxicity assay using splenocytes in the cured mice treated with combination therapy, and the cured mice rejected a second challenge of B16 melanoma cells.

Since HSPs function as natural and powerful immunostimulants, recombinant HSP70 therapy[64] or HSP70 gene therapy[65] could be a possible approach. For recombinant HSP70 therapy, MCLs and recombinant mouse HSP70 were injected into melanoma nodules in C57BL/6 mice, which were subjected to hyperthermia at 43°C for 30 min. The combined treatment strongly inhibited tumor growth over a 30-day period and complete regression of tumors was observed in 20% (2/10) of mice. It was also found that systemic antitumor immunity was induced in the cured mice. For HSP gene therapy, a human HSP70 gene mediated by cationic liposomes was injected into a B16 melanoma nodule in C57BL/6 mice *in situ*. At 24 h after the injection of the HSP70 gene, MCLs were injected into melanoma nodules in mice, which were subjected to hyperthermia at 43°C for 30 min. Tumor growth was strongly arrested over a 30-day period after the combined treatment. Complete regression of tumors was observed in 30% (3/10) of mice, and systemic antitumor immunity was induced in the cured mice. These results suggest that this novel therapeutic strategy, combining the use of HSPs with hyperthermia using MCLs, may be applicable to patients with advanced malignancies.

Conclusions

We have reviewed recent progress in immunology and immunotherapy based on MCL-induced hyperthermia via HSP expression. From the point of view of immunology, evidence has been accumulating that HSPs play an important role in the immune response after hyperthermia treatment. Further investigations to elucidate the mechanisms are ongoing. Our preliminary results indicate that the depletion of HSPs in heated-cell lysate decreased the CTL response. In addition, the decreased level of CTL response associated with HSP70 depletion was higher than that in HSP90 (unpublished results), suggesting that HSP70 plays a pivotal role in the immune response induced by hyperthermia. For immunotherapy, we demonstrated that some modalities enhanced the

immune response induced by hyperthermia. We are conducting further studies in order to establish a novel cancer immunotherapy based on the concept of heat-controlled necrosis with HSP expression.

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Extracellular heat shock proteins (eHSPs) pilot exogenous antigen into cross-presentation pathway: A superguide from extracellular world to intracellular tour

Yasuaki Tamura, Goro Kutomi, Jun Oura, Toshihiko Torigoe, Noriyuki Sato

Department of Pathlogy, Sapporo Medical University, School of Medicine

Abstract

Heat shock proteins (HSPs) are intracellular chaperone, some of which function as immune adjuvants as well as danger signals for immune system when released into extracellular milieu. These HSPs, along with their client polypeptides, are specifically bound by receptors on antigen presenting cells (APCs). This leads to APC differentiation along with delivery of the chaperoned peptides for cross-presentation to T cells. HSP-APC interactions occur through several receptors that mediate endocytosis or signal transduction. Most importantly, HSP associated antigens are forced to enter the cross-presentation pathway by APCs, resulting in $CD8^+$ T cell activation. These unique features of HSPs for the generation of immune response will be discussed.

Keywords: Heat shock protein, Hsp90, Cross-presentation, Antigen presenting cell, MHC class I molecule

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Introduction: HSP-mediated cross-presentation by antigen-presenting cells

It has been well demonstrated that immunization with tumor-derived HSPs or HSP complexed with an antigen peptide elicits tumor- or antigen-specific $CD8^+$ T cell responses. Above all, Hsp70- and gp96-antigen complexes are well-studied and have been shown to be immunogenic and potent in stimulating the generation of tumor-specific CTLs. Hsp70- and gp96-based vaccines have been tested in early-phase clinical trials in solid tumors as well as in lymphoma and leukemias; all showed minimal toxicity and potential efficacy[1-3]. Phase III clinical trials using tumor-derived Hsp70 and gp96 as vaccines are ongoing for melanoma and renal cell carcinoma.

The ability of HSPs to facilitate the cross-presentation of MHC class I-restricted epitopes and to prime $CD8^+$ T cell effector responses is well established[4,5]. Although, immunized HSPs are exogenous antigens, these HSP-antigen complexes can gain access to the class I antigen presentation pathway, resulting in cross-presentation. The immune response has been attributed to the ability of HSPs to form stable complexes with tumor-derived antigenic peptides, thereby facilitating the cross-presentation of MHC class I-restricted epitopes and priming of $CD8^+$ T cell responses.

Dendritic cells (DCs) are main conductor for efficient cross-presentation. Recent reports have shown that antigen-presenting cells (APCs) such as dendritic cells can internalize HSPs by receptor-mediated endocytosis and direct chaperoned proteins/peptides into the intracellular pathway for MHC class I-restricted presentation to $CD8^+$ T cells, concomitant with the induction of dendritic cell maturation and cytokine secretion. In fact, some HSP receptors expressed on APCs have recently been identified. CD91[6], LOX-1[7], CD40[8] and SR-A[9] have been proved to be common receptors for HSPs. However, the underlying mechanism for efficient cross-presentation, in particular, how the HSP-antigen complex can enter the MHC class I pathway, remains unclear.

Furthermore, recent studies have shown that HSP-peptide complexes can also lead to antigen presentation on MHC class II molecules, thus activating $CD4^+$ T cells[10,11]. Therefore, it is possible that uptake of HSP-peptides complexes leads to antigen presentation on both MHC class I and class II molecules on dendritic cells, thus activating $CD8^+$ CTL as well as $CD4^+$ T cells. However, Shild et al. have reported that, although antigen peptides chaperoned by gp96 can be presented in the context of both MHC class I and class II molecules, immunization with gp96 elicits CD8-biased T cell responses[12]. Doody et al have also demonstrated same results[5]. Therefore, it is essential to know the effects of the HSP-antigen complex on tumor antigen presentation via class

Correspondence to: Yasuaki Tamura, Department of Pathlogy, Sapporo Medical University, School of Medicine

I and class II pathways in vivo because such knowledge is crucial for the development of effective HSP-based immunotherapies, especially in the case of protein antigens in association with HSPs.

In contrast to Hsp70 and gp96, the role of Hsp90 in cross-presentation remains unclear. Hsp90 is the most abundant protein in the cytoplasm; therefore it is assumed that Hsp90 also plays an important role in cross-presentation. However, it is not clear whether Hsp90 is involved in tumor immunity. Udono reported, for the first time, that immunization with Hsp90 purified from tumor elicited tumor-specific CTL responses[13]. Very recently, Kunisawa and Shastri reported that Hsp90 chaperoned C-terminal flanking antigenic peptides[14]. These results have led to much interest in the importance of Hsp90 in antigen presentation. Taking these facts into consideration, it is conceivable that Hsp90 does participate in antigen presentation and possibly cross-presentation. In this paper, we will discuss the mechanism of HSP-mediated cross-presentation and the players involved in this intriguing immune response.

HSPs act as danger signals to the immune system through HSP receptors expressed on APCs.

It is suggested that extracellular HSPs (eHSPs) act as "danger signals" to the immune system in the case of life-threatening events (Fig. 1). When viral infection or tumor cell damage occurs, cell-associated antigens, such as HSPs-antigen complexes are released into the extracellular milieu, APCs such as dendritic cells and macrophages, detect the signals through certain receptors, resulting in intracellular signaling[15]. HSP receptors are divided into 2 categories, one is toll-like receptor (TLR) for mainly signaling for DC maturation and activation, and the other is endocytic receptors for cross-presentation. TLR-2 has been shown to be the receptor for Hsp70[16]. TLR-4 is the receptor for Hsp70[16,17]. However, doubts were raised as to what extent this effect was due to lipopolysaccharide contaminations of the HSP preparations. It is required re-examination for this phenomenon using HSPs, nominally endotoxin-free. In contrast, HSP-specific endocytic receptors expressed on the APCs, including CD91 for gp96, Hsp70 and Hsp90[6,18], SR-A for gp96 and calreticulin[9], LOX-1 for Hsp70[7], and CD40 for Hsp70[19], were identified. However, it is still unknown how HSP-antigen complexes are transported and where HSP releases chaperoned antigens. What is the fate of HSPs after endocytosis? What actors are responsible for translocating the antigen from the endosome to cytosol? Additional studies will be required to understand these unsolved issues.

The roads to MHC class I presentation

MHC class I molecules principally present peptides derived from endogenous protein to cytotoxic T cells. However, in certain antigen-presenting cells, peptides derived from exogenous antigens are also presented by MHC class I molecules. At least four independent pathways of protein processing and subsequent peptide presentation by class I molecules have been described. The dominant pathway uses endogenously synthesized proteins that have been processed in the cytosol by proteasomes. Peptides are transferred by transporter-associated antigen processing (TAP) to the ER where they bind in the grooves of nascent MHC class I molecules. The peptide/class I MHC heavy chain/β2m complex is then transported via the Golgi apparatus to the cell surface. In second and third pathways, exogenous antigens are internalized and processed into peptides that are transported to the ER to bind MHC class I. One is the cytosolic leak of internalized antigens by phagocytosis, macropinocytosis, and enocytosis, resulting in degradation by proteasomes[20,21]. Degraded peptides are then transported through TAP into the ER. However, the mechanism for the translocation of exogenous antigens to cytosol and players involved in this translocation remain unknown. Another pathway is via ER-phagosome fusion. When exogenous antigens are engulfed into phagosomes, the phagosomal membrane and ER membrane fuse with each other, forming ER-phagosome compartments[22,23]. These ER-phagosome fusion compartments involve TAP molecules and proteasomes outside of the membrane[24]. Phagocytosed antigens are pumped out the ER-phagosome fusion compartment through sec61. This is called the ER-associated degradation (ERAD) mechanism. Then proteasomes, which are attached to the outer face of the membrane of ER-phagosome fusion, degrade antigens into peptides, followed by entry into the ER again through TAP molecules, and the resulting antigen peptides bind to MHC class I molecules[25,26]. In contrast, at least some exogenous peptides or proteins appear to reach MHC class I through a pathway completely independent of the ER. In this fourth pathway, endosomal processing and endocytosed class I MHC molecules may be involved. MHC class I molecules have been shown to internalize from the cell surface in T cells, B cells, fibroblasts, and macrophages. Recycling of endocytosed class I MHC molecules back to the cell surface has also been observed[27]. The endocytosis and recycling of class I MHC may facilitate peptide exchange, allowing class I MHC molecules to bind multiple peptides in one lifetime. Antigen presentation mediated by the latter three types mentioned above is called cross-presentation, allows display of exogenous antigens in the context of MHC class I molecules. This is particularly important in

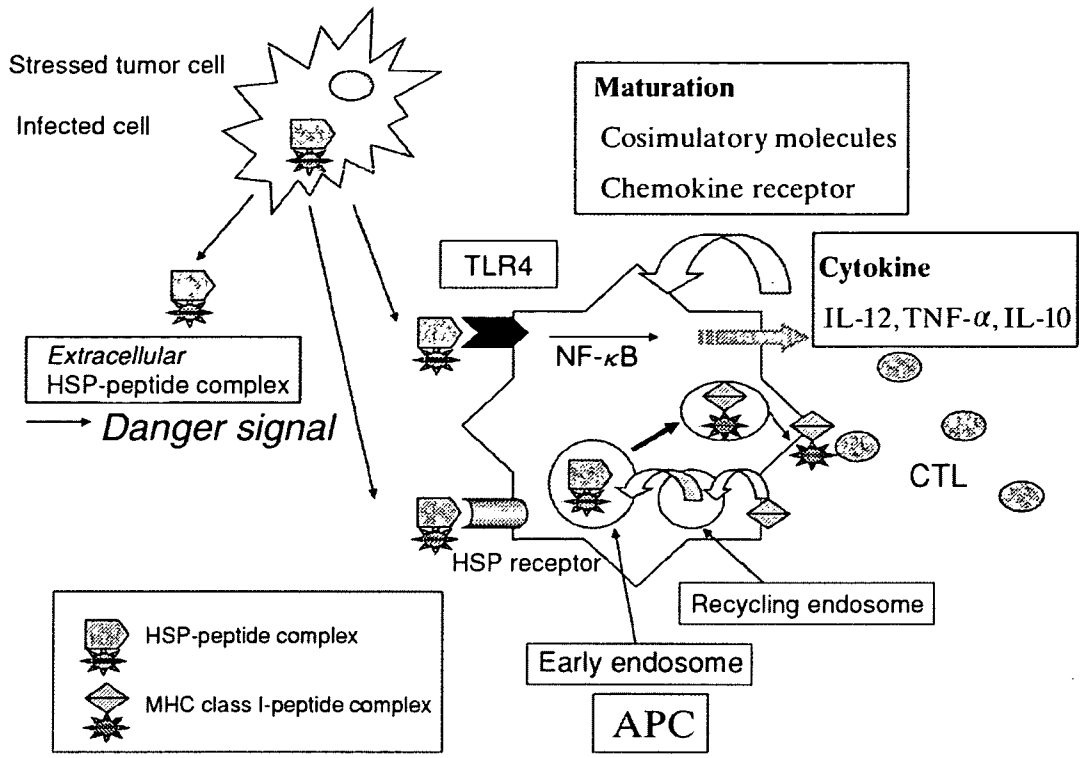

Fig. 1 **Role of extracellular HSP-peptide complex as a danger signal.** HSP-peptide complexes are acquired by bone marrow-derived antigen presenting cells (APC) and are cross-presented to cytotoxic T lymphocytes (CTL). HSP-peptide complexes bind to Toll-like receptor (TLR) 4 and induce maturation and activation of dendritic cells (DC). On the other hand, HSP-peptide complexes also bind to HSP receptor, such as CD91, LOX-1, SR-A, on the DC, followed by endocytosis. Internalized HSP-peptide complexes are shuttled into MHC class I pathway via endosomal pathway, and induce CTL response. HSP-peptide complexes elicit both innate and adaptive immunity simultaneously, indicating that HSP-peptide complexes act as danger signal.

host defense against infectious diseases and cancers that cannot access the classical pathway for MHC class I presentation. Moreover, for the development of the efficient cancer vaccine, it is necessary to establish the methods for delivering exogenous cancer vaccine into cross-presentation pathway.

Internalization of exogenous antigens may allow cell fragments, intracellular pathogens and proteins to be degraded in the endocytic pathway by mechanisms involving reduction, unfolding and lysosomal proteolysis. Such a process could contribute to cross-presentation by facilitating the exchange of previously loaded MHC class I-associated peptides for newly generated peptides derived from exogenous proteins. Cycloheximide treatment, which blocks the biosynthetic pathway of MHC I, indicated that the class I present in the endocytic compartments was derived from the cell surface. We have confirmed the presence of MHC class I in the endocytic compartments of murine bone marrow-derived DCs. Furthermore, as described recently, we have demonstrated that co-localization of the receptor-mediated endocytosed exogenous Hsp70/90-antigen complex with MHC class I in the early endosomes of the DCs. These observations suggest that antigens derived from the exogenous Hsp70/90 may be loaded onto recycling MHC class I, after which the MHC class I/peptide complex is transported to the cell surface.

Impact on HSP-antigen complex in a cross-presentation pathway

Considering the significance of the HSP-peptide complex in cross-presentation, this model suggests that a 'pre-processed antigen' would be required as it would be inefficient for a whole protein to be degraded non-specifically within the endocytic compartments by resident catabolic enzymes. Not only would this be a slow process, it would be by chance alone that an appropriate peptide capable of binding the MHC class I groove would be generated. The notion of such preprocessed antigens fits well with a role for HSPs as chaperones for peptide antigens. Proteins synthesized within the cell would be processed within the endogenous class I antigen presentation pathway leading to the generation of

HSP-peptide complexes. These complexes are ideal chaperones of antigenic peptides for the transfer of antigens to DCs for a number of reasons. Once generated within the cell, the HSP-peptide complex might be released into the extracellular milieu during cell necrosis because of viral infection and intervention of cancer, resulting in taking-up by the immature DC and acting as a danger signal. At the same time, antigenic peptides chaperoned by HSPs are efficiently presented in the context of MHC class I molecules and immediately activate the host's immune responses. As described earlier, Hsp90 binds precursor (pre-processed) peptides generated in the cells and thus, endosomal processing is a suitable mechanism for pre-processed peptides. HSP would also serve to protect the peptide antigen from degradation upon entry into the endocytic compartment of the DC. In addition, an as yet uncharacterized lysosomal enzyme may play a role in the processing of internalized antigens for generation of MHC I epitopes. Recently, Rock et al. have demonstrated that DC-restricted cathepsin S plays an important role in the processing of exogenous antigens for the generation of MHC I antigenic determinants in the early endosome[28].

Significance of cross-presentation in vivo: impact on epitope generation via endosomal processing and proteasomal processing

Although DCs are capable of using an endocytic exchange mechanism to create MHC class I-peptide complexes, typical somatic cells have only the classical pathway for the generation of MHC class I-presenting peptides. For $CD8^+$ T cells induced by cross-presentation to be functionally useful against pathogen-infected cells, it would seem that the epitope generation mechanism used should be the same as those in classical MHC class I processing. An endosomal exchange mechanism in which peptides are generated by different proteases in radically different conditions from those in the endogenous pathway, therefore seems unlikely to contribute substantially to the $CD8^+$ T cell repertoire. Cytoplasmic processing, including proteasomal proteolysis, and ER-based trimming would be expected to be involved to generate the same peptide sequences as those made by nonhematopoietic cells. In fact, although partial proteolysis may occur in the endocytic pathway, extensive experimental evidence suggests that exogenous antigens must reach the cytoplasm to be efficiently cross-presented. In DCs, the presentation of exogenous antigens is unaffected by both chloroquine and inhibitors of lysosomal proteolysis. Exogenous antigen presentation is, however, highly sensitive to specific inhibitors of the proteasome, indicating that cytoplasmic proteolysis is the main form of epitope generation in the cross-presentation pathway. In contrast, the HSP-mediated cross-presentation pathway has been shown to involve both a proteasomal pathway and an endocytic-recycling pathway. We have demonstrated that a tumor-derived Hsp70-peptide complex is efficiently cross-presented to peptide-specific CTLs by DCs and this presentation is dependent on TAP molecules. In addition, in vitro generated Hsp70- and gp96-antigen complexes have been shown to be cross-presented via a TAP-dependent parhway[29-31]. This fact suggests that processing and loading a peptide onto MHC class I requires translocation of the antigen from the endocytic compartment to the cytosolic pathway. Rodriguez et al. demonstrated that DCs have a unique membrane transport pathway linking the lumina of endocytic compartments and the cytosol[20]. Thus, in DCs, the exogenous HSP-chaperoned antigen in the endocytic compartment is released into the cytosol, where it follows the classical proteasome- and TAP-dependent class I pathway for presentation. Further studies to define the precise mechanisms for Hsp70- and Gp96-chaperoned peptide trafficking may reveal a new paradigm for cross-presentation.

Cross-presentation of exogenous Hsp90-peptide complex by dendritic cells

Hsp90 is a one of the most abundant proteins within cells and is overexpressed in many cancer cells. Therefore, once cancer cells become necrotic, much Hsp90 would be released from cells and might be a danger signal, subsequently eliciting cell-specific immune responses. It has been demonstrated that the tumor-derived Hsp90-peptide complex elicits tumor-specific immunity. At present, however, the processing pathway yielding the transfer of exogenous Hsp90-associated peptide antigens to MHC class I molecules is unknown.

We examined the roles of Hsp90 in MHC class I-restricted cross-presentation using bone marrow-derived dendritic cells (DCs) as APCs[32]. First, we tested whether Hsp90-peptide complexes reconstituted *in vitro* were taken-up and associated peptides presented in the context of MHC class I molecules by DCs. To monitor the MHC class I antigen-processing pathway, we used Hsp90 reconstituted *in vitro* with the C-terminal extended version of VSV8 (RGYVYQGL), VSV-C (RGYVYQGLKSGNVSC: 15mer) to moniter the processing of the precursor peptide. The Hsp90-VSV-C peptide complex was cocultured with DCs for 2 hours, followed by incubation with a VSV8-specific CTL clone. The culture supernatant was assayed for the production of IFN-γ. VSV-C-loaded Hsp90 was processed and presented by $H\text{-}2K^b$ and recognized by the VSV8-specific CTL clone, but not Hsp90 or VSV-C alone. In the presence of an anti-$H\text{-}2K^b$ mAb during the presentation assay, the presentation of VSV8 to the specific CTL clone was completely abolished. These data suggested that

Hsp90-bound VSV-C peptides were processed to VSV8 within the cells with subsequently gained access to the MHC class I pathway. Intriguingly, this presentation occurred within 15 min, indicating that very rapid and efficient processing might be achieved within DC (Fig.2A).

Next, we investigated whether the Hsp90-mediated MHC class I pathway required functional TAP molecules. To test this, we used DCs derived from the $TAP1^{-/-}$ mouse. Surprisingly, DCs from the $TAP1^{-/-}$ mouse could also process and present Hsp90-bound VSV-C peptides as efficiently as DCs from the wild-type mouse.

We also tested another well-characterized H-$2K^b$-restricted $OVA_{258-265}$ antigen system, Hsp90 reconstituted *in vitro* with the C-terminal extended version of SL8 ($OVA_{258-265}$), SL8-C peptide ($OVA_{258-270}$: 13mer). The Hsp90-SL8-C peptide complex was cocultured with DCs for 2 hours, followed by incubation with SL8-specific B3Z T cell hybridoma. As shown in Fig. 3B, the Hsp90-SL8C peptide complex was processed and presented by H-$2K^b$ and recognized by B3Z T cell hybridoma, but not Hsp90 or SL8-C alone, in a TAP-independent manner (Fig. 2B). These experiments demonstrate that a TAP-independent pathway is used for Hsp90-mediated MHC class I presentation.

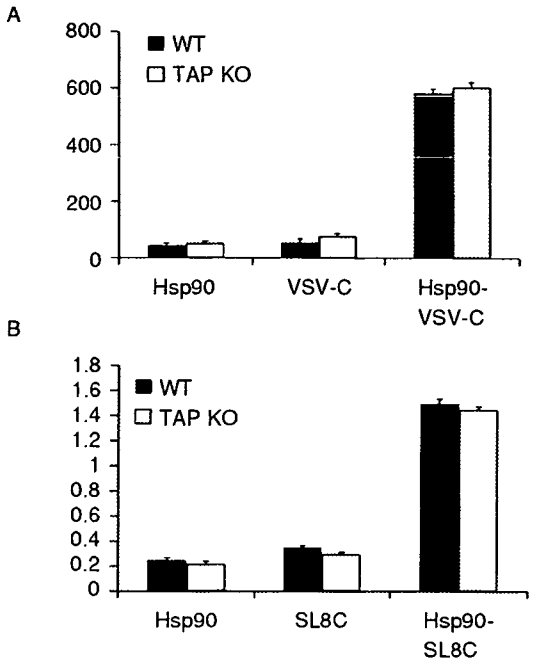

Fig. 2 **Precursor peptides chaperoned by Hsp90 are cross-presented via a TAP-independent pathway.** A. Hsp90-VSV-C complex, Hsp90 or VSV-C was loaded to BMDCs for 2 h and a VSV8-specific CTL clone was added. IFN-γ in the culture supernatant was measured by ELISA. BMDCs from the $TAP^{-/-}$ mouse could also process and present Hsp90-chaperoned VSV-C peptides efficiently as compared to BMDCs from the wild type mouse. B. SL8 precursor peptide, SL8C was also processed and cross-presented by BMDCs via a TAP-independent pathway.

Intracellular localization of endocytosed exogenous Hsp90-peptide complexes

Using laser confocal microscopy, we found that Hsp90-peptide complexes accumulated only in the endosome and did not reach the stage of the lysosome. We also examined whether Hsp90 accumulation in the endosome was due to temperature-dependent endocytosis. As expected, at 4°C, labeled Hsp90 remained on the cell surface, but internalization was evident after incubation at 37°C following a 10-min internalization period. According to a competition assay, DCs express Hsp90 receptor on the cell surfaces. Identification of receptor (s) will be necessary to elucidate the mechanism responsible for cross-presentation of exogenous Hsp90-antigen complexes.

The pathway for Hsp90-mediated cross-presentation

To investigate where the Hsp90-associated antigenic peptides bind to MHC class I molecules, we stained H-$2K^b$ molecules and exogenous Hsp90-peptide complexes. After 20 min of endocytosis, Alexa-labeled Hsp90 co-localized with endocytosed H-$2K^b$ molecules. The results showed the internalization and co-localization of H-$2K^b$ and Hsp90 was evident in the early endosome. This finding suggested that HSP-bound peptides might be transferred to MHC class I molecules in the endosome, where recycled MHC class I molecules from the plasma membrane are available. The peptide-MHC class I complexes generated in the endosome are then transported to the cell surfaces of the DCs, where specific CTLs recognize them.

Recycling of endocytosed MHC class I molecules back to the cell surface has been observed. Some of the recycled MHC class I molecules can be loaded into endosomes with peptides derived from endocytosed molecules[27]. Therefore, to confirm whether this presentation really utilizes recycled MHC class I molecules, we treated DCs with primaquine, which blocks the membrane recycling pathway. DCs incubated in the presence of this drug could not present the Hsp90-chaperoned SL8C-derived SL8 peptide. This result indicated that precursor peptides chaperoned by Hsp90 or processed peptides entered into recycling endosomes and transferred onto recycling MHC class I molecules, which returned to the cell surface and stimulated B3Z T cell hybridoma. To analyze the involvement of vacuolar acidification of endosomal compartments, DCs were incubated with Hsp90-SL8C in the presence of chloroquine, a known inhibitor of acidification of endosomal compartments.

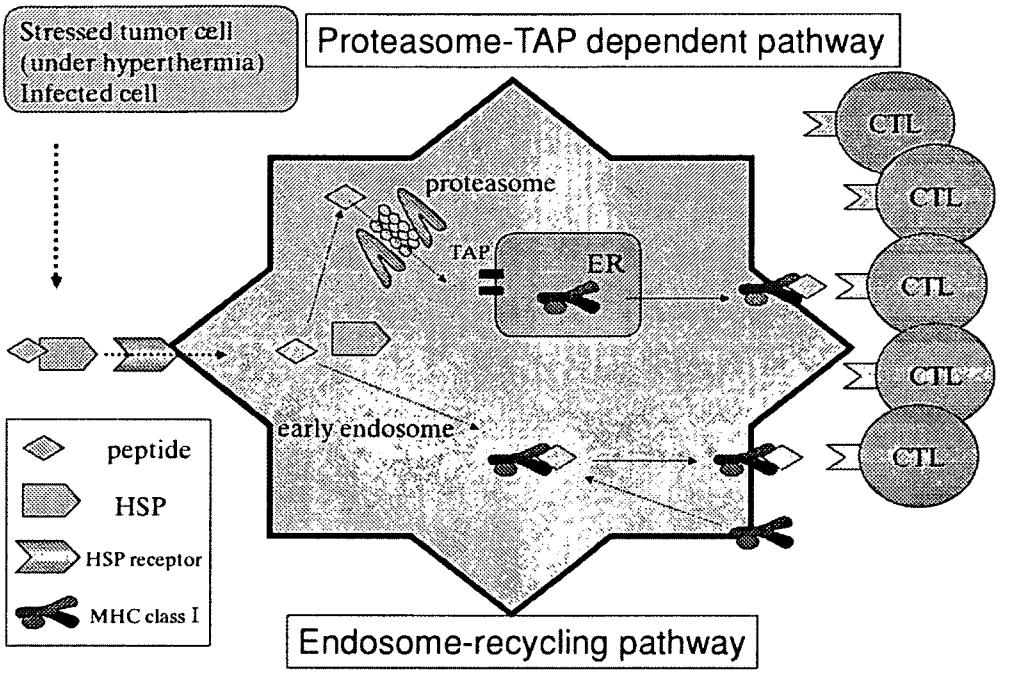

Fig. 3 **Possible pathway of Hsp90-antigen complex-mediated cross-presentation.** Internalized Hsp90-antigen complexes through receptor-mediated endocytosis follow 2 distinct MHC class I pathways. (1) Internalized Hsp90-antigen complexes are transferred to the cytosol and imported into the ER in a TAP-dependent fashion. Hsp90-chaperoned antigens are degraded in the cytosol by proteasome and further trimmed by cytosolic peptidases. The resulting peptides are transported into the lumen of the ER for loading on newly synthesized MHC class I molecules. (2) Alternatively, internalized antigens chaperoned by Hsp90 are processed and loading in the endocytic pathway onto MHC class I molecules that are recycled from the plasma membrane, independently of TAP.

Chloroquine strongly inhibited Hsp90-mediated presentation without affecting SL8 peptide presentation. Thus, acidification of endosomal compartments is necessary for processing of Hsp90-chaperoned precursor peptides (Fig. 3).

Hsp90-chaperoned precursor peptides are processed by the endosomal protease

We used protease inhibitors to investigate the proteolytic processes involved in the Hsp90-mediated TAP-independent cross-presentation pathways. In wild-type DCs, a broadly active cysteine protease inhibitor, leupeptin, almost completely inhibited the cross-presentation of Hsp90-SL8C. In contrast, the aspartic protease inhibitor pepstatin did not affect the cross-presentation. Cathepsins S, D and L are known to be the major cysteine proteases in endocytic compartments. We therefore examined the roles of various cathepsins in this pathway. Cathepsin D- and cathepsin L-specific inhibitors did not affect the cross-presentation, whereas a cathepsin S inhibitor completely blocked cross-presentation. Cathepsin S is a cysteine protease that is preferentially expressed in APCs, including DCs, macrophages, and B cells within endocytic compartments. Therefore, our data indicate that cathepsin S is a critical enzyme in TAP-independent Hsp90-mediated cross-presentation on MHC class I molecules and that the presented peptides are indeed generated in endosomal compartments.

Advantages of Hsp90-antigenic protein complexes

As described above, we have shown that Hsp90-chaperoned precursor peptides are efficiently processed and presented by MHC class I molecules. To extend the range of HSP-based immunotherapy, we examined whether whole protein antigens chaperoned by Hsp90 were processed and presented by MHC class I molecules as well as class II molecules. The advantages of using protein antigens for cancer immunotherapy are that they can (1) provide an inherent polyvalent vaccine for $CD8^+$ T cells, and (2) they include $CD4^+$ helper epitopes, required for efficient CTL induction and proliferation. However, protein antigens themselves are not primarily immunogenic and therefore an immunostimulatory adjuvant is necessary for effective T cell responses. Given the well-known ability of HSP to forms complexes with naturally synthesized proteins, it is possible that Hsp90-protein antigen complexes could elicit antigen-specific

CTL responses and Th responses as well. Therefore, we investigated the impact of Hsp90 on the presentation of exogenous protein antigens using OVA protein as a model antigen. We observed that the Hsp90-OVA antigen complex generated in vitro was very efficiently and selectively presented via the MHC class I pathway both in vitro and in vivo. Surprisingly and unexpectedly, we observed that the cross-presentation of Hsp90-OVA complexes was involved both TAP-dependent and -independent pathways, unlike the results of Hsp90-precursor peptide complex. These results provide a rationale for the development of novel vaccination strategies for cancer immunotherapy.

Hsp90-OVA complex is efficiently cross-presented by DCs.

We evaluated cross-presentation of the Hsp90-OVA protein complex. DCs were pulsed with Hsp90 alone, free OVA, a simple mixture of the two or the two in a complex generated in vitro for 2 hrs at 37°C, then fixed, washed and cultured with B3Z $CD8^+$ T cell hybridoma. The Hsp90-OVA complex elicited strong CTL responses, whereas Hsp90 or OVA alone did not lead to CTL responses. Notably, when we pulsed the simple mixture of Hsp90 and OVA, we did not detect significant CTL responses. These results show that binding to Hsp90 is essential for cross-presentation of OVA.

Hsp90-OVA complex is efficiently and preferentially presented through MHC class I, but not class II pathway

We also tested whether the Hsp90-OVA complex was presented through the MHC class II pathway, and elicited $CD4^+$ T cell responses. DC from B6C3F1 were pulsed with free OVA or Hsp90-OVA complex for 2 hrs at 37°C, then fixed, washed and co-cultured with B3Z $CD8^+$ T cell hybridoma or KZO $CD4^+$ T cell hybridoma. Stimulation with free OVA led not to CTL responses but strong $CD4^+$ T cell responses. In contrast, stimulation with the Hsp90-OVA complex elicited significantly weaker $CD4^+$ T cell responses than free OVA, whereas it induced robust CTL responses. These findings suggest that the Hsp90-OVA complex is presented much more selectively through the MHC class I pathway than the MHC class II pathway.

To examine the differences in presentation efficacy between the Hsp90-OVA complex and free OVA, a pulse-chase experiment was performed. DC were pulsed with OVA alone or the Hsp90-OVA complex at 37°C, and harvested at different times from 10 min to 2 hrs, then fixed, washed and cultured with B3Z or KZO. B3Z responses were seen after 10 to 30 min of stimulation with the Hsp90-OVA complex, although no KZO responses were detected up to 1 hr with free OVA. These data demonstrate that cross-presentation of the Hsp90-OVA complex is more rapid and efficient than presentation of free OVA.

Why and how do HSP-antigen complexes skew the $CD8^+$ T cell responses but not $CD4^+$ T cell responses?

Ramirez et al. reported that a gp96-peptide complex elicited $CD8^+$ T cell responses but not $CD4^+$ T cell responses[12]. We have also observed that presentation of exogenous Hsp90-chaperoned peptides and protein antigens tends to drive the $CD8^+$ T cell response. Although, protein antigens such as OVA protein contain both a $CD8^+$ T cell epitope and $CD4^+$ T cell epitope, if OVA is chaperoned by Hsp90, the Hsp90-OVA complex is taken up by receptor-mediated endocytosis and enters the cross-presentation pathway, followed by $CD8^+$ T cell responses. In contrast, the soluble form of OVA protein is pinocytosed and follows the classical class II pathway. As for what drives Hsp90-antigen complexes into the cross-presentation pathway, the regulators for this transport are unclear and further studies will be required to elucidate the precise mechanism.

The mechanism for translocation of Hsp90-chaperoned antigens from endosomes to cytosol.

The mechanism for antigen escaping to the cytosol remains unknown, as is whether the Hsp90-antigen complex or its components separately escape to the cytosol. It is possible that the Hsp90-antigen complex first needs to be preprocessed in the endosomal compartments before being transferred to the cytosol to be further degraded by proteasomes. One possibility is that the mildly acidic pH in the endocytic compartments plays an important role for the transport of ingested antigens, and another is that delayed fusion with the late endosome/lysosome is important for the transport. Immature DCs maintained mildly acidic pH in the endocytic compartment even after antigen uptake and could transport these antigens into the cytosol[21]. Chloroquine treatment inhibits the acidification of the endocytic compartments. Our data indicated that chloroquine treatment inhibited Hsp90-OVA presentation by DCs in both TAP-dependent and -independent pathways. This suggested that antigen transport was dependent on mildly acidic pH-inducible machinery in the endocytic compartments of DCs. However, a recent report showed that treatment with chlorquine or NH_4Cl enhanced the efficiency of cross-presentation[33]. These treatments accelerated export of exogenous soluble antigens from endocytic compartments to cytosol, thereby enhancing cross-presentation. The difference between

The presentation pathway of Hsp90-protein complexes

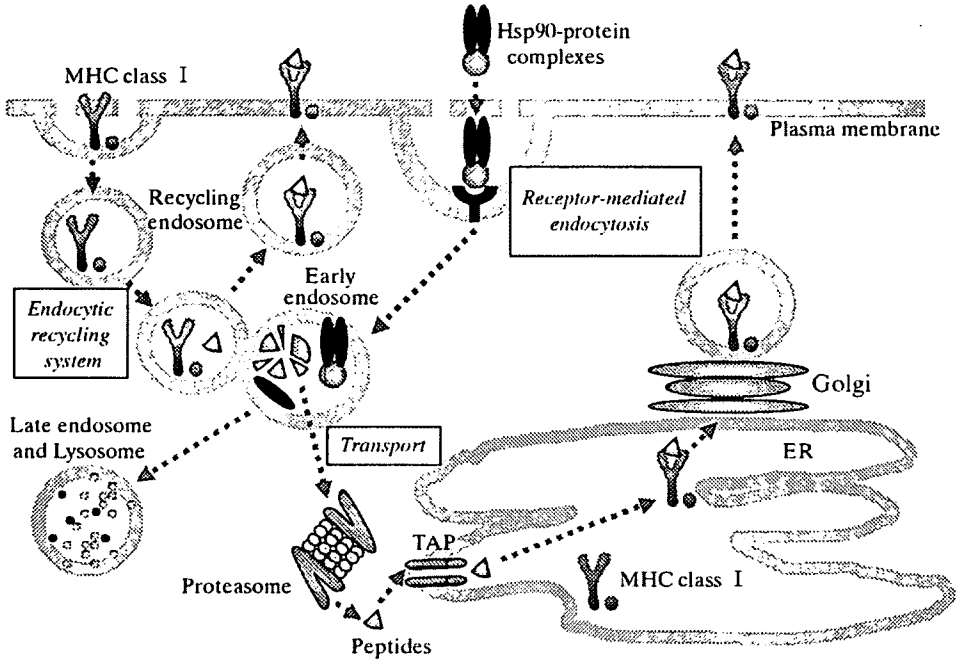

Fig. 4 **Route of access to the cytosol for internalized Hsp90-antigen complexes.** Pathways for generation of Hsp90-chaperoned peptide-MHC class I complexes. Antigens are first cleaved endosomal protease, such as cathepsin S, and resulting peptide intermediates then enter into cytosol, followed by proteasomal degaradation. A small fraction of peptide intermediates are further trimmed by endosomal protease within endosomes, thereafter reach recycling endosomes and are loaded onto MHC class I molecules, which the return to plasma membrane occurs. It remains unclear what the mechanisms are by which they traverse the endosomal membrane or if it reflects the existence of a specific channel or a translocator.

our results and theirs was the method of uptake by DCs. In our case it occurred via receptor-mediated endocytosis, whereas they indicated that it occurred via phagocytosis or macropinocytosis. In addition, the regulators for the transport are still unclear. We are still on the road to complete understanding of HSP-mediated immune regulation, and further studies will be required to elucidate the precise mechanism (Fig. 4).

Conclusion

Although HSPs are primarily cytosolic proteins, they play an important role as a "danger signal" in the extracellular milieu on behalf of immune surveillance. Above all, Hsp90 is one of the most abundant cytosolic proteins and elicits intriguingly efficient and rapid CTL responses. In this meaning, Hsp90 is a "smart and excellent guide" for the MHC class I cross-presentation pathway. A forthcoming issue is to elucidate the mechanism of the driving force toward the $CD8^+$ T cell response mediated by HSPs. In addition, how endocytosed HSP-chaperoned antigens are translocated to be processed? These findings will clarify the impact of HSP as a danger signal in the etiologies of autoimmune diseases and tumor immunity.

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