Heat shock protein 90 targets a chaperoned peptide to the static early endosome for efficient cross-presentation by human dendritic cells

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The presentation of an exogenous antigen in a major histocompatibility complex class-I-restricted fashion to CD8+ T cells is called cross-presentation. Heat shock proteins (HSPs) such as Hsp70, gp96, and Hsp90 have been shown to elicit efficient CTL responses by cross-presentation through an as-yet entirely unknown mechanism. Hsp90 is the most abundant cytosolic HSP and is known to act as a molecular chaperone. We have shown that a tumor antigen peptide complexed with Hsp90 could be cross-presented by dendritic cells (DCs) through an endosomal pathway in a murine system. However, it has not been determined whether human DCs also cross-present an Hsp90–peptide complex and induce peptide-specific CTLs. In this study, we found that an Hsp90–cancer antigen peptide complex was efficiently cross-presented by human monocyte-derived DCs and induced peptide-specific CTLs. Furthermore, we observed that the internalized Hsp90–peptide complex was strictly sorted to the Rab5–EEA1+ static early endosome and the Hsp90-chaperoned peptide was processed and bound to MHC class I molecules through an endosome-recycling pathway. Our data indicate that targeting of the antigen to a “static” early endosome by Hsp90 is essential for efficient cross-presentation.

The generation of specific CD8+ CTLs is thought to play a key role in the control of virus-infected cells and tumors. However, immunization with peptides or recombinant proteins generally fails to elicit CTLs because an immunized antigen (Ag) acts as an exogenous Ag. Generally, an exogenous Ag enters the MHC class II pathway and is presented to CD4+ T cells in the context of MHC class II molecules. However, professional Ag-presenting cells, particularly DCs, can take up exogenous Ag and present them on their MHC class I molecules. This process is called cross-presentation and plays an important role in the control of virus-infected cells and tumor growth.1(3) There are two pathways of cross-presentation: cytosolic (endoplasmic reticulum–Golgi-dependent) and vacuolar (endosomal) pathways.2(3) One of the reasons for inefficiency of a vaccine strategy is that the vaccine Ag is usually administered as an exogenous Ag, and it is therefore difficult to introduce the vaccine Ag into the cross-presentation pathway. To overcome this problem, various methods have been developed to target an exogenous Ag into the endogenous MHC class I-restricted pathway. In our previous studies, we showed that extracellular Hsp90–peptide complexes are efficiently cross-presented through the endosome-recycling pathway.4(5) In this Hsp90-mediated cross-presentation, the receptor-dependent endocytosed Hsp90–peptide complex was transferred to the early endosome in which a cysteine protease such as cathepsin S processed the precursor peptide. The resulting MHC class I epitope was transferred onto recycling MHC class I molecules, thereby resulting in the expression of an MHC class I–epitope complex on the cell surface. Furthermore, we have shown that immunization with Hsp90–tumor Ag peptide complexes induces Ag-specific CTL responses and strong antitumor immunity in vivo. However, how the Hsp90–peptide complex is sorted out after receptor-dependent endocytosis remains unclear. In the present work, we found that Hsp90 complexed with a human tumor Ag peptide derived from survivin-2B5(6) is cross-presented by human Mo-DCs resulting in the stimulation of peptide-specific CTLs. In addition, we found that Hsp90 targets a chaperoned Ag peptide into the “static” early endosome within Mo-DCs, resulting in cross-presentation of the antigenic peptide through the recycling pathway.

Materials and Methods
The study protocol was approved by the Clinic Institutional Ethical Review Board of the Medical Institute of Bioregulation, © 2014 The Authors. Cancer Science published by Wiley Publishing Asia Pty Ltd on behalf of Japanese Cancer Association. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
Sapporo Medical University (Sapporo, Japan). The patients and their families as well as healthy donors gave informed consent for the use of blood samples in our research.

**Patient treatment.** The patients were vaccinated with survivin-2B90.88 (1 mg) plus Montanide ISA 51 (1 mL; Seppic, Paris, France) s.c. four times at 14-day intervals. In addition, IFN-α (3 000 000 IU; Dainippon-Sumitomo Pharmaceutical Co., Osaka, Japan) was given s.c. twice a week close to the site of vaccination. Hematological examinations were carried out before and after each vaccination.

**Induction of human monocyte-derived immature dendritic cells.** Autologous monocytes were purified from PBMCs from each patient that were isolated using Lymphoprep (Nycodren, Oslo, Norway). Monocytes (1 × 10^7/well) in a 24-well plate were cultured in complete RPMI-1640 with 10% FCS and GM-CSF (1000 U/mL) and IL-4 (1000 U/mL) for 7 days. The medium with GM-CSF and IL-4 was gently replaced on day 2 and 4. Human recombinant GM-CSF (PromoCell, Heidelberg, Germany) and IL-4 (Peprotech) were added at 100 ng/mL and 1000 U/mL, respectively.

**Peptides and proteins.** The following peptides were used (underlined sequences representing the predicted MHC class I binding epitope): survivin-2B90.88 (AYACNTSTL), and survivin-2B75.93 (GPGTVAYACNTSTLGRGG). All peptides were purchased from guidedsynosys (Ishigaki, Japan). Human Hsp90 was purchased from StressGen (Ann Arbor, MI, USA). Human LDL was purchased from Sigma-Aldrich (St. Louis, MO, USA) and stored at 20 mg/mL in PBS at −80°C.

**Antibodies.** Confocal laser microscopy was used to detect organelles with specific antibodies: an anti-RAβ3 pAb (MBL, Nagoya, Japan) and EEA1 (Abcam, Cambridge, MA, USA) for early endosomes, and anti-LAMP-1 pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for late endosomes/lysosomes. Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA) was used for labeling Hsp90 and LDL.

**Generation of Hsp90-peptide complex in vitro.** As previously described, Hsp90 was mixed with survivin-2B75.93 (GPGTVAYACNTSTLGRGG) in a 50:1 peptide:protein molar ratio in 0.7 M NaCl containing sodium-potassium buffer and heated at 45°C for 30 min, then incubated for 30 min at room temperature.

**Establishment of survivin-2B90.88-specific CTL clone.** We generated survivin-2B90.88-specific CTL clones from a patient with colon cancer (patient 1 in Table 1). After the fourth vaccination, PBMCs were isolated from blood samples by Ficoll-Conray density gradient centrifugation. Phytohemagglutinin blasts were derived from PBMCs by cultivating in AIM V medium (Invitrogen) containing 10% human serum, IL-2 (100 U/mL; Takeda Pharmaceutical Co., Osaka, Japan), and PHA (1 mg/mL; Wako Chemicals, Osaka, Japan) for 3 days, followed by washing and cultivation in the presence of IL-2 (100 U/mL) for 4 days. HL-A2.4204-survivin-2B90.88 peptide tetramer-positive (MBL) CTLs were sorted and subsequently cloned to single cells using FACSAria (Becton Dickinson, San Jose, CA, USA). Survivin-2B90.88-specific CTL clones were restimulated with survivin-2B90.88 peptide-pulsed PHA blasts every 7 days in AIM V medium supplemented with 50 U/mL IL-2.

**In vitro cross-presentation assay.** Human Mo-DCs (1 × 10^4) were pulsed with Hsp90 (400 μg/mL), survivin-2B90.88 (400 μg/mL) alone, a complex of Hsp90 (100 or 400 μg/mL) and survivin-2B75.93 (100 or 400 μg/mL), a simple mixture of both or survivin-2B90.88 (400 μg/mL) for 2 h at 37°C in 100 μL Opti-MEM and then fixed for 1 min with 0.01% glutaraldehyde.

Fixation was stopped by addition of 2 M L-lysine and the cells were washed twice with RPMI-1640 medium and cultured overnight with 1 × 10^5 survivin-2B peptide-specific CTL clone. Activation of CTLs was measured as IFN-γ production using ELISA. In a dose titration assay, Mo-DCs (1 × 10^5) were loaded with various doses of survivin-2B90.88 peptide or Hsp90-precursor peptide (survivin-2B75.93) complex for 2 h in 100 μL Opti-MEM and fixed with 0.01% glutaraldehyde. The cells were washed and cultured overnight with 1 × 10^5 survivin-2B90.88 peptide-specific CTL clone. Interferon-γ in the culture supernatant was measured using ELISA.

**In vitro stimulation of PBMCs with Mo-DC loaded with Hsp90-precursor peptide complex.** Peripheral blood mononuclear cells were isolated from eight patients suffering from various types of cancer who had been vaccinated with survivin-2B peptide in our clinical study. These patients’ PBMCs were shown to contain survivin-2B-specific CD8+ T cells. The PBMCs were stimulated with Mo-DCs loaded with survivin-2B90.88 (400 μg/mL), Hsp90 (400 μg/mL), survivin-2B75.93 (400 μg/mL), and Hsp90 (100 or 400 μg/mL)–survivin-2B75.93 (100 or 400 μg/mL) complex in AIM V medium (Life Technologies, Grand Island, NY, USA) containing 10% human serum. Interleukin-2 was added at a final concentration of 50 U/mL on days 2, 4, and 6. On day 11 of culture, the PBMCs were analyzed by tetramer staining and ELISPOT assay.

**Assessment of stimulation of Ag-specific CTLs using tetramer assay.** The FITC-labeled HLA-A*-2402-HIV peptide (RYSRDQQLL) and PE-labeled HLA-A*-2402-survivin-2B90.88 tetramers were purchased from MBL. For flow cytometric analysis, PBMCs, which were stimulated in vitro as described above, were stained with HIV tetramer or survivin-2B tetramer and CD8-positive T cells were analyzed using FACSCalibur and CellQuest software. The CD8+ living cells were gated and cells labeled with survivin-2B tetramer were referred to as tetramer-positive cells. The frequency of CTL precursors was calculated as the number of tetramer-positive cells divided by the number of CD8+ T cells.

**Assessment of stimulation of Ag-specific CTLs using ELISPOT assay.** The ELISPOT plates were coated steriley overnight with anti-IFN-γ capture antibody (BD Biosciences, San Jose, CA, USA) at 4°C. The plates were then washed once and blocked with AIM V medium containing 10% human serum for 2 h at room temperature. CD8-positive T cells separated from patients’ PBMCs (5 × 10^5 cells/well), which were stimulated in vitro as described above, were then added to each well along with HLA-A2-transfected T2 (T2-A24) cells (5 × 10^5 cells/well) that had been preincubated with survivin-2B90.88 (10 μg/mL) or HIV with an HIV peptide as a negative control. After incubation in a 5% CO2 humified chamber at 37°C for 24 h, the wells were washed vigorously five times with PBS and incubated with a biotinylated anti-human IFN-γ antibody (R&D Systems, Minneapolis, MN, USA) and HRP-conjugated avidin. Spots were visualized and analyzed using KS ELISPOT (Carl Zeiss, Jena, Germany).

**Immunocytological localization of Hsp90–survivin-2B75.93 peptide complex.** Heat shock protein 90 and LDL were conjugated with Alexa Fluor 594 (Molecular Probes) according to the manufacturer’s instructions. Monocyte-derived DCs were incubated at 37°C with Alexa Fluor 594-labeled Hsp90 (20 μg) complexed with survivin-2B75.93 peptide (20 μg) for 1 h.
Following incubation, cells were washed twice with ice-cold PBS and fixed with ice-cold acetone for 1 min. Organelles were stained with an anti-Rab5 pAb and EEA1 mAb for early endosomes and anti-LAMP-1 pAb for late endosomes followed by Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) or anti-mouse IgG (Molecular Probes) and then visualized with a Bio-Rad MRC1024ES confocal scanning laser microscope system (Bio-Rad, Richmond, CA, USA). For evaluation of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells from three independent experiments) were analyzed. Inhibition studies. Monocyte-derived DCs were pre-incubated with chloroquine (Sigma-Aldrich) or primaquine (ICN Biomedicals, Irvine, CA, USA) at 37°C for 2 h, and then loaded with survivin-2B80-88 peptide alone or Hsp90–precursor peptide (survivin-2B75-93) complex for 2 h. The Mo-DCs were then fixed, washed, and cultured overnight with survivin-2B80-88–specific CTL clone. Activation of CTLs was measured as IFN-γ production using ELISA.

Statistical analysis. All experiments were independently carried out three times in triplicate. Results are shown as means ± SEM. Comparisons between two groups were performed using Student’s t-test, with a P-value < 0.05 considered to be statistically significant.

Results

Heat shock protein 90–survivin-2B75-93 peptide complex is cross-presented by Mo-DCs in vitro. We first examined whether human Hsp90 facilitated cross-presentation of the chaperoned precursor peptide by human Mo-DCs. The Mo-DCs were pulsed with Hsp90 alone, the survivin-2B75-93 precursor peptide alone, a simple mixture of both, a complex of them generated in vitro at double concentration, or survivin-2B80-88 peptide (for positive control) for 2 h at 37°C and then fixed, washed, and cultured with survivin-2B80-88–specific CTL clone. The Hsp90–survivin-2B75-93 precursor peptide complex elicited a significant amount of IFN-γ production both at 100 and 100 μg/mL, whereas Hsp90 alone, survivin-2B75-93 precursor peptide alone, or a simple mixture of both did not induce IFN-γ production by CTLs (Fig. 1a). Strikingly, IFN-γ production induced by Hsp90–survivin-2B precursor peptide complex was much greater than that induced by survivin-2B peptide. These results indicated that cross-presentation of survivin-2B-derived peptide was enhanced when an exogenous precursor peptide was complexed to Hsp90. To confirm these observations, we compared the efficacy of CTL activation between survivin-

Table 1. Quantitation of survivin-2B-specific CD8+ T cells by tetramer assay

| Patient no. | Tumor   | Survivin-2B80-88-specific CD8+ T cell frequency (tetramer staining) | Survivin-2B75-93 peptide complex | Effect  
|-------------|---------|------------------------------------------------------------------|---------------------------------|---------
|             | In vitro stimulation | (-) | Survivin-2B80-88 | Hsp90 | Survivin-2B75-93 | Hsp90–survivin-2B75-93 | Effect |
| 1           | Colon   | 0.06 | 4.87 | 4.47 | 3.24 | 8.47 | †† |
| 2           | Colon   | 0.32 | 2.87 | 0.77 | 4.01 | 1.30 | No |
| 3           | Pancreas | 0.70 | 6.27 | 1.70 | 1.88 | 6.64 | †† |
| 4           | Pancreas | 0.48 | 4.56 | 0.84 | 1.28 | 3.02 | †† |
| 5           | Ampulla of Vater | 1.27 | 4.60 | 0.97 | 2.24 | 6.50 | †† |
| 6           | Breast  | 3.59 | 3.78 | 3.00 | 3.06 | 3.82 | †† |
| 7           | Breast  | 3.98 | 3.91 | 1.94 | 2.28 | 6.19 | †† |
| 8           | Breast  | 2.76 | 3.92 | 2.91 | 2.08 | 6.07 | †† |

†† Frequency of survivin-2B-specific CD8+ T cells stimulated with heat shock protein 90 (Hsp90)–survivin-2B75-93 peptide complex was increased compared with stimulation with survivin-2B75-93 Precursor peptide.  *Frequency of survivin-2B-specific CD8+ T cells stimulated with Hsp90–survivin-2B75-93 peptide was increased compared with stimulation with both survivin-2B80-88 peptide and survivin-2B75-93 peptide. (-), negative control; No, no effect.
2B80-88 peptide and Hsp90–survivin-2B75-93 precursor peptide complex in a dose titration assay (Fig. 1b). We observed that stimulation of the survivin-2B90-93-specific CTL clone with Hsp90–survivin-2B75-93 precursor peptide complex was more effective than stimulation with survivin-2B80-88 peptide at any dose.

Peptide-specific precursor CTLs are activated by cross-presentation of Hsp90–peptide complex. As we had shown that the Hsp90–survivin-2B75-93 precursor peptide complex was efficiently cross-presented, we next examined whether cross-presentation of Hsp90–peptide complex could activate and expand peptide-specific memory CD8+ T cells from patients who had been vaccinated with survivin-2B peptide with incomplete Freund’s adjuvant. Activated and expanded survivin-2B-specific CD8+ T cells were detected by tetramer staining. As shown in Figure 2, the survivin-2B75-93 precursor peptide chaperoned by Hsp90 was able to activate and expand survivin-2B-specific memory CD8+ T cells more vigorously than was the precursor peptide alone. Interestingly, peptide-specific T-cell frequency was higher when stimulated with Hsp90–survivin-2B75-93 precursor peptide complex than that with survivin-2B80-88 peptide, indicating that a long peptide chaperoned by Hsp90 was efficiently cross-presented and was able to stimulate peptide-specific CD8+ T cells. To confirm these observations, we compared the efficacy of activation of survivin-2B-specific memory CD8+ T cells by stimulation with survivin-2B80-88, survivin-2B75-93 precursor peptide, or Hsp90–survivin-2B75-93 precursor peptide complex in eight patients. As shown in Table 1, stimulation with Hsp90–survivin-2B75-93 complex could expand survivin-2B-specific memory CD8+ T cells from seven out of eight patients compared with stimulation with survivin-2B80-88, survivin-2B75-93 precursor peptide complex.

Memory CD8+ T cells activated by cross-presentation of Hsp90–peptide complex become functional peptide-specific CTLs. To further confirm whether survivin-2B-specific CD8+ T cells activated by Hsp90-mediated cross-presentation were functional or not, we carried out an ELISPOT assay using CD8+ T cells from a patient who had been vaccinated with survivin-2B peptide with incomplete Freund’s adjuvant. Figure 3 shows that stimulation of CD8+ T cells from the patient with Hsp90–survivin-2B75-93 precursor peptide complex clearly increased functionally positive survivin-2B-specific CD8+ T cells compared with stimulation with survivin-2B75-93 precursor peptide or survivin-2B80-88 peptide. When CD8+ T cells from the patient were stimulated with Hsp90 (400 μg/mL)–survivin peptide (400 μg/mL) complex, the number of IFN-γ-positive spots was less than that of CD8+ T cells stimulated with Hsp90 (100 μg/mL)–survivin peptide (100 μg/mL) complex. These results were due to the formation of fused large spots that were observed when stimulated with Hsp90 (400 μg/mL)–survivin peptide (400 μg/mL) complex and therefore the number of ELISPOT counted became smaller than that of Hsp90 (100 μg/mL)–survivin peptide (100 μg/mL) complex. These findings indicated that Hsp90–peptide complex is efficiently cross-presented by human Mo-DCs and is capable of stimulating peptide-specific CTLs.

Immunocytochemical localization of Hsp90–survivin2B75-93 peptide complex. For further support of the above-described results, we investigated the intracellular routing of Hsp90 after uptake of it in DCs, using confocal laser microscopy. The Mo-DCs were incubated with Alexa 594-labeled Hsp90–survivin2B75-93 peptide complex for 1 h. Following incubation, the cells were fixed and stained with antibodies against markers for organelle structures including EEA1, Rab5, and LAMP-1. Alexa 594-labeled Hsp90–peptide complex was detected in EEA1+ and Rab5+ early endosomes but not in lysosomes (Fig. 4a). Quantitative analysis of the colocalization between the exogenous Hsp90–peptide complex and Rab5, EEA1, and LAMP1 revealed average colocalization incidences of 78.0%, 88.7%, and 7.3%, respectively, providing further evidence that the exogenous Hsp90–peptide complex was delivered to the endosome-recycling pathway (Fig. 4b). We also examined the dynamics of Alexa 594-labeled LDL as a positive control protein for the dynamic early endosomal pathway (Fig. 5). Alexa594-labeled soluble LDL localized to the Rab5+ early endosome as well as the LAMP-1+ late endosome/lysosome.
but not to the EEA1⁺-compartment, thus indicating the dynamic endosomal pathway. These results indicated that the Hsp90–peptide complex was sorted into the static endosomal pathway, not the dynamic endosomal pathway, within human Mo-DCs. In contrast, the soluble LDL protein, which underwent degradation, was translocated to the dynamic endosomal pathway. These results suggested that targeting to the “static” early endosome was required for efficient cross-presentation by Mo-DCs.

Heat shock protein 90–peptide complex is cross-presented by human DCs through an endosome-recycling pathway. We then examined whether Hsp90–precursor peptide complex was cross-presented by human Mo-DCs through an endosomal pathway after targeting to the static early endosome. We used chloroquine for inhibition of endosomal acidification and primaquarine for inhibition of the membrane recycling pathway. As shown in Figure 6(a), Mo-DCs that were pre-incubated with increasing concentrations of chloroquine completely blocked cross-presentation of Hsp90–survivin-2B75-93 precursor peptide complex but had no substantial effect on survivin-2B80-88 peptide presentation. These results indicated that cross-presentation of Hsp90-precursor peptide complex depended on endosomal acidification, possibly including proteolysis by endosomal proteases. Moreover, Mo-DC incubated with primaquarine could not present the Hsp90-chaperoned precursor peptide-derived survivin-2B80-88 peptide to CTL (Fig. 6b). These results indicated that the Hsp90-chaperoned precursor peptide or processed peptide entered recycling endosomes and were transferred onto recycling MHC class I molecules.

Discussion

It has been shown that immunization with tumor-derived HSPs or HSPs complexed with an Ag peptide/protein elicits tumor-specific CD8⁺ T cell responses. Importantly, it has been shown that Hsp70–Ag and gp96–Ag complexes facilitate Ag presentation in association with MHC class I molecules. Recently, we and Calderwood’s group have shown that Hsp90 also acted as an excellent navigator for associated antigens to enter the cross-presentation pathway in the murine system. We here showed that human Hsp90–cancer Ag peptide complex was efficiently cross-presented by human Mo-DCs. These results hold promise for the development of a safe and efficient immunomodulator for cancer immunotherapy. More importantly, we showed that translocation of the Hsp90–Ag complex into the static early endosome after endocytosis was crucial for efficient cross-presentation. It has been shown that the pathway for cross-presentation is comprised of two distinct intracellular routes, a proteasome–TAP-dependent pathway and an endosome-recycling pathway. Recent studies have revealed the pathway in which peptide exchange onto recy-

![Image](https://www.wileyonlinelibrary.com/journal/cas)
clinging MHC class I molecules occurs within early endosomal
compartments.\(^{(15)}\) We have shown that Hsp90–peptide complex-mediated\(^{(4)}\) and ORP150–peptide complex-mediated\(^{(16)}\) cross-presentation was independent of TAP and was sensitive
to primaquine, indicating that sorting of peptides onto MHC
class I occurs through an endosome-recycling pathway. Lakad-
amyali et al.\(^{(17)}\) have shown that early endosomes are com-
prised 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. Cargos destined for degradation, including LDL,
epidermal growth factor, and influenza virus, are internalized
and targeted to the Rab5+, EEA1–dynamic population of early
endosomes as we have observed using LDL, thereafter traffick-
ing to Rab7+-late endosomes. In contrast, the recycling ligand
transferrin is delivered to Rab5+, EEA1–static early endo-
somes after internalization by Mo-DCs, suggesting that preferential sorting to the “static” endosome is
necessary for cross-presentation of Hsp90–peptide complexes.
In contrast, soluble LDL protein was targeted to the EEA1
and LAMP-1+-dynamic early endosome–late endosome/lyso-
some pathway, leading to degradation and presentation in the
context of MHC class II molecules. These findings suggested
that Hsp90 shuttled the chaperoned precursor peptide into the
static endosome-recycling pathway, preventing further degrada-
tion, followed by transfer of the peptide onto recycling MHC
class I molecules. Together, our findings indicate that the role

![Fig. 5. Low-density lipoprotein (LDL) was targeted to the dynamic early endosome followed by translocation to the late endosome/lysosome for degradation.](image)

![Fig. 6. Heat shock protein 90 (Hsp90)–peptide complex is cross-presented through an endosome-recycling pathway.](image)
of Hsp90 in cross-presentation is to navigate the associated Ag into static early endosomes within human Mo-DCs. Thus, Hsp90 appears to be a promising natural immunomodulator for use of cancer vaccine development due to its excellent ability to target human DCs and to induce specific CTLs.

Disclosure Statement
The authors have no conflict of interest.

Abbreviations
Ag antigen

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