Heat shock enhances the expression of cytotoxic granule proteins and augments the activities of tumor-associated antigen-specific cytotoxic T lymphocytes

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Received: 1 February 2012 / Revised: 19 June 2012 / Accepted: 21 June 2012 / Published online: 11 July 2012
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Abstract Focal inflammation causes systemic fever. Cancer hyperthermia therapy results in shrinkage of tumors by various mechanisms, including induction of adaptive immune response. However, the physiological meaning of systemic fever and mechanisms of tumor shrinkage by hyperthermia have not been completely understood. In this study, we investigated how heat shock influences the adaptive immune system. We established a cytotoxic T lymphocyte (CTL) clone (#IM29) specific for survivin, one of the tumor-associated antigens (TAAs), from survivin peptide-immunized cancer patients' peripheral blood, and the CTL activities were investigated in several temperature conditions (37–41 °C). Cytotoxicity and IFN- γ secretion of CTL were greatest under 39 °C condition, whereas they were minimum under 41 °C. To address the molecular

mechanisms of this phenomenon, we investigated the apoptosis status of CTLs, expression of CD3, CD8, and TCR $\alpha\beta$ by flow cytometry, and expression of perforin, granzyme B, and Fas ligand by western blot analysis. The expression of perforin and granzyme B were upregulated under temperature conditions of 39 and 41 °C. On the other hand, CTL cell death was induced under 41 °C condition with highest Caspase-3 activity. Therefore, the greatest cytotoxicity activity at 39 °C might depend on upregulation of cytotoxic granule proteins including perforin and granzyme B. These results suggest that heat shock enhances effector phase of the adaptive immune system and promotes eradication of microbe and tumor cells.

Keywords Heat shock · CTL · Perforin · Survivin

Electronic supplementary material The online version of this article (doi:10.1007/s12192-012-0348-0) contains supplementary material, which is available to authorized users.

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Abbreviations

CTL	Cytotoxic T lymphocyte
TAA	Tumor-associated antigen
HLA	Human leukocyte antigen
PBMC	Peripheral blood mononuclear cell
mAb	Monoclonal antibody
HSP	Heat shock protein

Introduction

Inflammation is defined by four classical signs, pain (dolor), heat (calor), redness (rubor), and swelling (tumor), caused by secretion of inflammatory cytokines (e.g., IL-1 β , TNF α , and IL-6) from inflammatory cells (Bernheim et al. 1979). Inflammatory cytokines induce the secretion of prostaglandin E2 from endothelial cells of the central nervous system, and prostaglandin E2 acts in the hypothalamic area, resulting in fever. Although fever is one of the well-described symptoms of

Table 1 Summary of antibodies

Antigen	Company	Clone	Dilution	Application
CD3	Beckman Colettr	UCHT1		Flow cytometry
CD8	BD	SK1		Flow cytometry
TCR $\alpha\beta$	Thermo scientific	BMA031		Flow cytometry
HLA-A24	— ^a	C7709A2.6	Culture sup.	Flow cytometry
Fas Ligand	MBL	CH-11	1000	Western blot
Perforin	SIGMA	3B4	1000	Western blot
Granzyme B	R&D systems	351927	1000	Western blot
HSP90	Enzo Life Sciences	AC88	1000	Western blot
HSP70	Enzo Life Sciences		1000	Western blot
Fas Ligand	R&D systems	#154922	1000	Western blot
β -actin	SIGMA	AC15	2000	Western blot

^aKind gift from Dr. P. G. Coulie

inflammation, the physiological meanings on adaptive immunity involving cytotoxic T lymphocytes (CTLs) still remain unclear.

Hyperthermia for cancer treatment has been of clinical interest for many years, though the exact anti-tumor mechanism remains unclear. Several reports have indicated that heat treatment enhanced adaptive immune response by CTL cross-priming due to induction of heat shock proteins (HSPs). HSPs associated with antigenic peptides and HSPs induced by heat treatment in cancer cells enhance cancer-specific adaptive immune responses by cross-priming (Bachleitner-Hofmann et al. 2006; Brusa et al. 2009; Sato et al. 2009, 2010; Shi et al. 2006; Torigoe et al. 2009). On the other hand, the direct effects of heat treatment on established CTLs remain unknown.

In this study, we investigated the effect of heat treatment on a CTL clone specific for survivin, one of the tumor-associated antigens (TAAs; Hirohashi et al. 2002). Heat treatment (39 °C) of CTLs enhanced the cytotoxicity and the secretion of IFN- γ of CTLs, whereas heat treatment with higher temperature (41 °C) abrogated CTL functions. To address the molecular mechanism of this phenomenon, we examined changes in the expression of several molecules in CTLs and found that the expression of cytotoxic granule proteins (perforin and granzyme B) is enhanced by heat treatment. On the other hand, the expression of CD3, CD8, and TCR B did not show any difference. The viability of CTLs was abrogated and apoptotic cells were increased by higher heat treatment (41 °C). These findings indicate that heat treatment of CTLs at 39 °C enhances CTL functions

Fig. 1 Establishment of CTL clone #IM29. **a** Schematic summary of establishment of CTL clone. Survivin tetramer-positive cells were single-cell-sorted into a 96-well plate at single cell per well. The specificity of growing T cells was assessed by survivin- and HIV-tetramer staining. **b** Cytotoxicity of CTL clone #IM29. Cytotoxicity of CTL clone #IM29 was evaluated with survivin peptide-pulsed T2-A24 cells, control peptide (HIV)-pulsed T2-A24 cells and K562 cells. Asterisks represent significant difference compared with HIV peptide-pulsed T2-A24 cells or K562 cells ($P < 0.05$, t test). Data are mean $\pm$ SD

partially by upregulation of perforin and granzyme B, and that heat treatment of CTLs at 41 °C abrogates CTL functions partially by inducing apoptosis. Augmentation of CTL functions by heat treatment might be one significant reason of fever induced by inflammation and also one mechanism of hyperthermia.

Materials and methods

Cells and antibodies

T2-A24 cells, human leukocyte antigen (HLA)-A24 gene-transduced T2 cells, were a kind gift from Dr. K. Kuzushima (Nagoya, Japan). The cells were cultured in RPMI1640 (SIGMA) supplemented with 10 % FBS (Life Technologies) and 800 µg/ml of G418 (Life Technologies). K562 cells were obtained from ATCC and

cultured in RPMI1640 supplemented with 10 % FBS. The antibodies used in this study are summarized in Table 1.

Establishment of CTL clone #IM29

A survivin peptide-specific CTL clone was established from peripheral blood of a 44-year-old patient with rectal carcinoma who was treated with survivin peptide immunization and IFN α (Kameshima et al. 2011). Survivin peptide-specific CTLs were stained with survivin peptide-HLA-A24 complex tetramer and were single-cell-sorted by FACS Aria II (BD) into a round-bottomed 96-well plate at single cell per well. The CTL was incubated with irradiated 8×10^4 peripheral blood mononuclear cells (PBMCs) in AIM-V (Life Technologies) medium supplemented with 10 % human AB serum (kindly provided by Dr. Tatsuo Usui, Hokkaido Red

Fig. 2 Heat treatment enhances CTL functions. **a** Cytotoxicity of CTL clone #IM29 in several temperature conditions. Cytotoxicity of CTL clone #IM29 using T2-A24 cells pulsed with survivin peptide was evaluated by LDH release assay under several temperature conditions (37, 39, and 41 °C). Asterisks represent significant difference ($P < 0.05$, t test). Data are mean $\pm$ SD. **b** IFN- γ secretion of CTL clone #IM29 using T2-A24 cells pulsed with survivin peptide was evaluated by ELISA under several temperature conditions (37, 39, and 41 °C). Data are mean. **c** Cytotoxicity of CTL clone #IM29 using aCella TOX assay.

Cytotoxicity of CTL clone #IM29 using T2-A24 cells pulsed with survivin peptide was evaluated by aCella TOX assay under several temperature conditions (37, 39, and 41 °C). Asterisks represent significant difference ($P < 0.05$, t test). Data are mean $\pm$ SD. **d** Cytotoxicity of CTL clone #IM29 in 39 °C condition. CTLs were preincubated in 39 °C for 1 day before cytotoxicity assay. Cytotoxicity of CTL clone #IM29 was evaluated with survivin peptide-pulsed T2-A24 cells, control peptide (HIV)-pulsed T2-A24 cells and K562 cells in 39 °C. Asterisks represent significant difference compared with HIV peptide-pulsed T2-A24 cells or K562 cells ($P < 0.05$, t test). Data are mean $\pm$ SD

Cross Blood Center, Sapporo, Japan), 200 IU of IL-2 (R&D Systems), and 5 µg/ml of PHA (SIGMA). Growing wells were transferred into a 24-well plate and fed every 3 days with AIM-V supplemented with 10 % human AB serum and 200 IU of IL-2. On day 34, clones were assessed by survivin tetramer staining and HIV-tetramer as a negative control (Fig. 1a). Tetramers were obtained from MBL Co., Ltd. (Nagoya, Japan).

Cytotoxicity assay and IFN-γ ELISA

CTL clone #IM29 were preincubated at 37–41 °C for 1 day before cytotoxicity assay and IFN-γ ELISA. Survivin peptide-pulsed T2-A24 cells were seeded into a 96-well plate at 5×10^3 cells/well. CTL clone #IM29 cells were seeded at several effector/target (*E/T*) ratios, then incubated at 37–41 °C for 6 h. Cytotoxicity of CTL clone #IM29 cells was examined by using an LDH cytotoxicity detection kit (TAKARA BIO Inc., Osaka, Japan) and aCella TOX kit (Cell Technology, Inc., Mountain View, CA) according to the manufacturer's protocol.

Ten thousand survivin peptide-pulsed T2-A24 cells and 3×10^3 of CTL clone #29 cells were co-cultured in a 96-well plate at 37–41 °C for 12 h, and then IFN-γ concentrations in supernatants were measured by using a Human IFN gamma ELISA Kit (Thermo Scientific) as described in the manufacturer's protocol.

Live images of cytotoxicity were recorded by the OLYMPUS FLUOVIEW FV 300-I71BG-SP system (OLYMPUS). Briefly, T2-A24 cells were transduced with GFP-plasmid using a Nucleofector V kit (Amaxa). GFP-positive T2-A24 cells were pulsed with survivin peptide and co-cultured with CTL clone #IM29 cells at an *E/T* ratio=10, and cultured in 37–41 °C for 4 h and live image were recorded.

Flow cytometry

For detection of HLA-A24 of T2-A24 cells, T2-A24 cells were stained with anti-HLA-A24 mAb (C7709A2.6, kind gift from Dr. P. G. Coulie, Brussels, Belgium) for 1 h, washed three times with PBS, stained with FITC-labeled anti-mouse IgG+M antibody (KPL, 200 times dilution) for 30 min, and washed again one time with PBS. T2-A24 cells were analyzed by a FACS Calibur (BD).

After treatment of CTL clone #IM29 cells at 37–41 °C for 12 h, the expression of CD3, CD8, and TCRαβ was examined by ad FACS Calibur. After staining with first antibodies (summarized in Table 1), CTL clone #IM29 cells were stained with FITC-labeled anti-mouse IgG+M antibody and then analyzed by a FACS Calibur. Mean fluorescent intensity was calculated by CELL Quest software (BD).

Western blot

Western blot analysis was performed as described previously (Nakatsugawa et al. 2011). Anti-perforin, granzyme B, Fas ligand, HSP90, and β-actin mAbs were used at 1,000×, 1,000×, 1,000×, 1,000×, and 2,000× dilutions, respectively (summarized in Table 1). The specific bands were quantified by using Image J software (NIH).

Detection of apoptosis

Apoptotic cells were detected by staining with anti-annexin V antibody and propidium iodide (PI) using annexin V FLUOD staining kit (Roche) according to the manufacturer's protocol and then analyzed by a FACS Calibur. For detection of dead cells, CTLs were stained with Trypan Blue (Life Technologies) and % dead cells were calculated by Countess Automated Cell Counter (Life Technologies). Caspase-3 activity was measured by using APOPCYTO Caspase-3 Colorimetric Assay Kit (MBL, Nagoya, Japan) according to the manufacturer's protocol. CTLs were preincubated under 37–41 °C conditions for 24 h, before caspase-3 assay.

Fig. 3 Heat shock did not alter HLA-A24 expression on T2-A24 cells. The expression of HLA-A24 on T2-A24 cells under several temperature conditions was evaluated by a FACS Calibur. Mean fluorescent intensity (MFI) are listed in ESM 5

Results

Establishment of survivin-derived antigenic peptide-specific CTL clone

To establish a CTL clone specific for cancer cells, we sorted survivin peptide-specific CTLs using survivin peptide-

HLA-A24 complex tetramer (Fig. 1a). Sorted CTLs were grown in 96-well plates and a survivin tetramer-positive CTL clone (#IM29) was established (Fig. 1a). CTL clone #IM29 recognized survivin peptide-pulsed T2-A24 cells at different *E/T* ratios, whereas it did not recognize control peptide-pulsed T2-A24 cells or K562 cells, indicating that CTL clone #IM29 is specific for survivin peptide (Fig. 1b).

Fig. 4 Expression of several molecules of CTL in several temperature conditions. **a** Flow cytometer analysis of surface molecules on CTL clone #IM29 cells under several temperature conditions. CD3, CD8 and TCRαβ expression on CTL clone #IM29 under several temperature conditions was analyzed by using a flow cytometer. Mean fluorescent intensity (MFI) are listed in ESM 6. **b** Western blot analysis of CTL clone #IM29 under several temperature conditions. Perforin, granzyme B, Fas ligand and HSP90 protein expression in CTL clone #IM29 under several temperature conditions was analyzed by western blot analysis. β-Actin was used as a

internal positive control. Data are representative Western Blot pictures. **c** Quantification of protein expression in CTL clone #IM29 under several temperature conditions. Western blot bands were analyzed, and quantified by Image J software (NIH). Perforin, granzyme B, Fas ligand, and HSP90 expression were standardized by β-Actin. Data are relative expression of perforin, granzyme B, Fas ligand and HSP90. **d** Heat shock did not alter Fas expression on T2-A24 cells. The expression of Fas on T2-A24 cells under several temperature conditions was evaluated by a FACS Calibur. Mean fluorescent intensity (MFI) are listed in ESM 7

Heat treatment enhanced CTL functions

To address the effects of heat treatment on CTL functions, we evaluated CTL functions under several temperature (37–41 °C) conditions (Fig. 2a, b, c and [Electronic supplementary material \(ESM\)](#)). Cytotoxicity of CTL was significantly enhanced in 39 °C condition compared with 37 °C condition, whereas the cytotoxicity was decreased in 41 °C condition by LDH release assay (Fig. 2a). IFN- γ secretion also showed similar pattern as cytotoxicity with highest IFN- γ secretion in 39 °C condition (Fig. 2b). To confirm the cytotoxicity results using LDH release assay, we performed aCella TOX assay. aCella TOX assay also showed that the cytotoxicity of CTL was greatest in 39 °C condition, and minimum in 41 °C (Fig. 2c). The specificity of CTL was not altered in 39 °C condition indicating that heat shock enhances only antigenic peptide-specific cytotoxicity, but not non-specific cytotoxicity (Fig. 2d).

Heat shock induced the HSP70 expression in T2-A24 cells ([Supplemental data 4](#)), whereas the expression of HLA-A24 molecule on T2-A24 cells was not enhanced under heat shock conditions (Fig. 3).

Heat treatment upregulated the expression of perforin

CTLs recognize target cells through cell surface molecules, including CD3, CD8, and TCR $\alpha\beta$, and kill target cells by secretion of cytotoxic granule proteins including perforin and granzyme B. We investigated the expression of these molecules in CTLs under several temperature conditions (Fig. 4a and b). The expression of CD3, CD8, and TCR $\alpha\beta$ on the surface of CTLs were almost the same in all temperature conditions (37–41 °C; Fig. 4a and b). On the other hand, the expression of perforin and granzyme B were enhanced in high temperature conditions (39 °C and 41 °C) compared with its expression in 37 °C condition (Fig. 4b and c). Fas ligand protein, another cytotoxic molecule expression and Fas expression on T2-A24 cells did not show any increase (Fig. 4b and d).

Heat treatment (41 °C) increased apoptotic cell death of CTLs

Since CTL activity was minimum in the condition of 41 °C, we investigated the cell conditions of CTLs in several

Fig. 5 Apoptotic cell death was induced under 41 °C condition. **a** Detection of apoptosis under several temperature conditions. Apoptotic cell death was detected by anti-annexin V and propidium iodide (PI) staining under several temperature conditions. Percentages represent apoptotic cells (annexin V-positive and PI-negative cells). **b** Percent dead cells was increased in 41 °C. CTLs were preincubated in 37–41 °C

for 1 day. CTLs were stained with Trypan Blue and %dead cells were counted by Countess Automated Cell Counter. Asterisks represent significant difference ($P<0.05$, t test). Data are mean+SD. **c** Caspase 3 was activated in 41 °C. CTLs were preincubated in 37–41 °C for 1 day. Caspase 3 activities were measured by APOPCYTO Caspase-3 Colorimetric Assay Kit. Data are mean+SD

temperature conditions. Trypan Blue staining of CTLs cultured in several temperatures revealed that the percentage of dead cells was highest in 41 °C condition (Fig. 5b). To address the type of cell death, annexin V and PI staining was performed to detect apoptotic cell, which were greater at 41 °C than in other temperature conditions (Fig. 5a); and Caspase-3 activity was highest in the 41 °C condition (Fig. 5c). Thus, the CTL cell death partially by apoptosis under 41 °C condition might be the reason of low CTL activity in high temperature.

Discussion

The physiological meaning of fever caused by inflammation has been a major issue for a long time. In this study, we showed for the first time that CTL activity was upregulated by heat treatment. Results of the cytotoxicity assay and IFN- γ assay showed that CTL activity was greatest at 39 °C and that it was the minimum at 41 °C. These observations indicate that fever caused by systemic inflammatory cytokine release enhances CTL functions, and this might enhance eradication of pathogens. The enhancement of cytotoxicity might depend on upregulation of cytotoxic granule proteins including perforin and granzyme B. After secretion from CTLs, perforin is inserted into the plasma membrane, make pore and lyse target cells. Upregulation of cytotoxic granule proteins might not be involved in the enhancement of IFN- γ secretion, suggesting the existence of another molecular mechanism to enhance CTL functions by heat shock.

The exact molecular mechanisms by which cytotoxic granule proteins are upregulated in heat-treated CTLs remain unclear. Since we treated CTLs at 39 °C, it may be transcription enhanced due to the activation of heat shock factor-1 (HSF1); however, there is no HSF1-binding site (heat shock element) in the promoter region of perforin and granzyme B. (Pipkin et al. 2010) Another possible explanation is stabilization of perforin protein by heat treatment. Heat treatment induces upregulation of several chaperone proteins including HSPs by HSF1, and thus association with these chaperone proteins may stabilize and prolong the life span of cytotoxic granule proteins. Further analysis is needed to clarify the exact molecular mechanisms.

Hyperthermia is one treatment modality for cancers, but the exact mechanisms by which it works to suppress cancers remain unclear. Adaptive immune systems induced by antigenic peptides bound to HSPs might be mechanisms (enhancement of induction phase of CTL). In this study, we showed that heat treatment enhanced the cytotoxicity activity of CTLs (enhancement of effector phase of CTL). Therefore, heat treatment will

enhance both induction and effector phases of adaptive immune system. These two mechanisms should act synergistically, and the adaptive immune system should have a role in hyperthermia.

In summary, we showed for the first time that heat treatment of CTLs enhanced CTL functions. Upregulation of cytotoxic granule proteins may play a role in this phenomenon. Enhancement of CTL functions by heat treatment might have a role in CTL functions in fever and hyperthermia.

Acknowledgments The authors thank Ms. E. Nakazawa for technical assistance. The authors thank Dr. Tatsuo Usui (Hokkaido Red Cross Blood Center, Sapporo, Japan) for the kind donation of human sera. The authors thank Dr. K. Kuzushima and Dr. P. G. Coulie for providing cells. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant Nos. 16209013, 17016061 and 15659097) for Practical Application Research from the Japan Science and Technology Agency, and for Cancer Research (15-17 and 19-14) from the Ministry of Health, Labor and Welfare of Japan, Ono Cancer Research Fund (to N. S.) and Takeda Science Foundation (to Y. H.). This work was supported in part by the National Cancer Center Research and Development Fund (23-A-44).

Declaration of financial disclosure The authors have no financial conflict of interest.

References

- Bachleitner-Hofmann T et al (2006) Heat shock treatment of tumor lysate-pulsed dendritic cells enhances their capacity to elicit anti-tumor T cell responses against medullary thyroid carcinoma. *J Clin Endocrinol Metab* 91:4571–4577
- Bernheim HA et al (1979) Fever: pathogenesis, pathophysiology, and purpose. *Ann Intern Med* 91:261–270
- Brusa D et al (2009) Immunogenicity of 56 degrees C and UVC-treated prostate cancer is associated with release of HSP70 and HMGB1 from necrotic cells. *Prostate* 69:1343–1352
- Hirohashi Y et al (2002) An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. *Clin Cancer Res* 8:1731–1739
- Kameshima H et al (2011) Immunogenic enhancement and clinical effect by type-I interferon of anti-apoptotic protein, survivin-derived peptide vaccine, in advanced colorectal cancer patients. *Cancer Sci* 102:1181–1187
- Nakatsugawa M et al (2011) SOX2 is overexpressed in stem-like cells of human lung adenocarcinoma and augments the tumorigenicity. *Lab Invest* 91:1796–1804
- Pipkin ME et al (2010) The transcriptional control of the perforin locus. *Immunol Rev* 235:55–72
- Sato N et al (2009) Molecular pathological approaches to human tumor immunology. *Pathol Int* 59:205–217
- Sato A et al (2010) Melanoma-targeted chemo-thermo-immuno (CTI)-therapy using *N*-propionyl-4-*S*-cysteaminylphenol-magnetite nanoparticles elicits CTL response via heat shock protein–peptide complex release. *Cancer Sci* 101:1939–1946
- Shi H et al (2006) Hyperthermia enhances CTL cross-priming. *J Immunol* 176:2134–2141
- Torigoe T et al (2009) Heat shock proteins and immunity: application of hyperthermia for immunomodulation. *Int J Hyperthermia* 25:610–616