図4

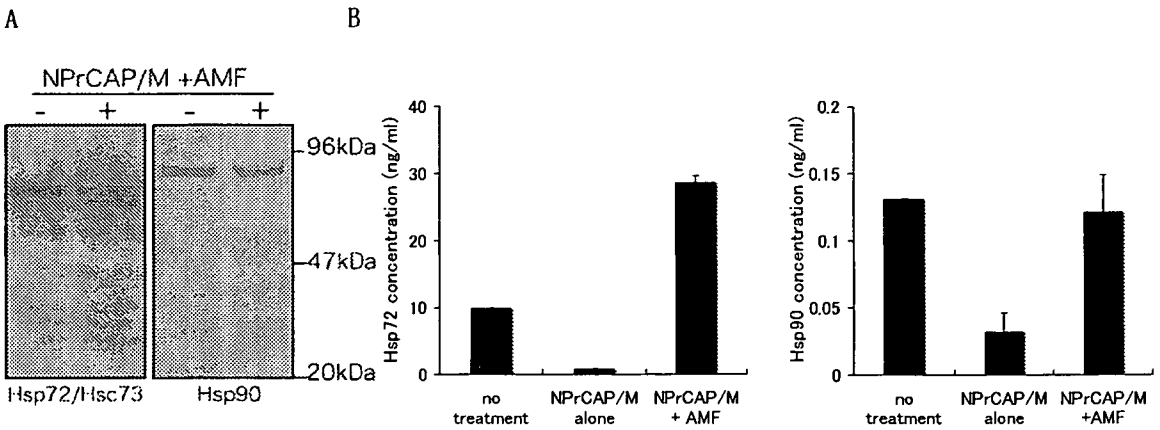


図5

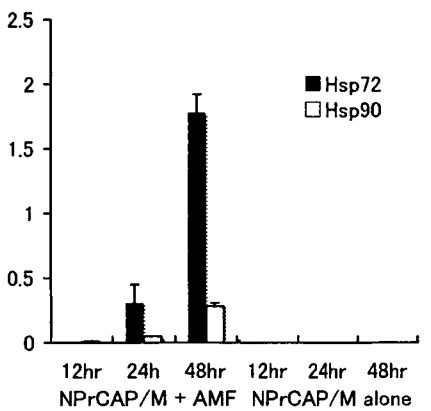


図6

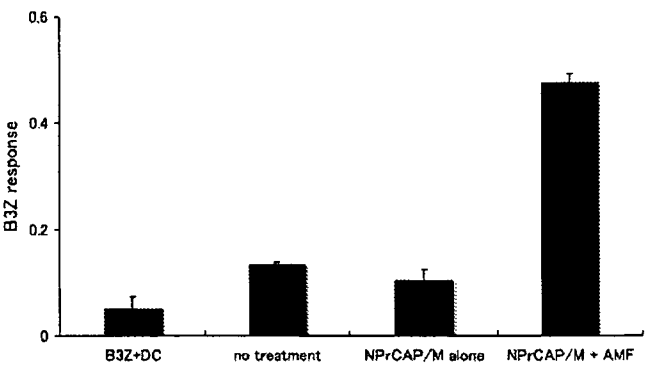
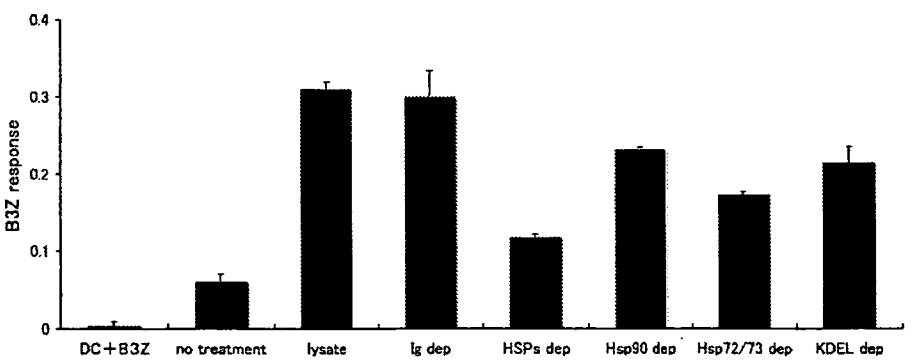
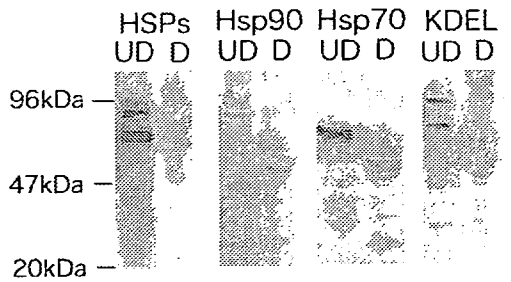


図7



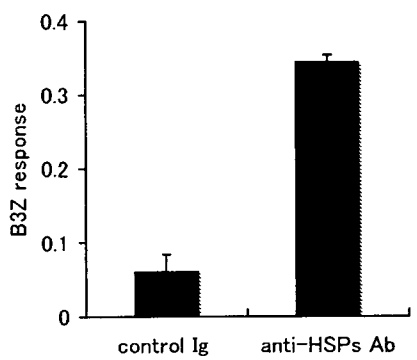
A. HSP 除去後の細胞抽出液をパルスした樹状細胞と **B3Z** の反応

図 7B



HSP 除去前（UD）と除去後（D）の細胞抽出液の western blotting

図 8 A



B

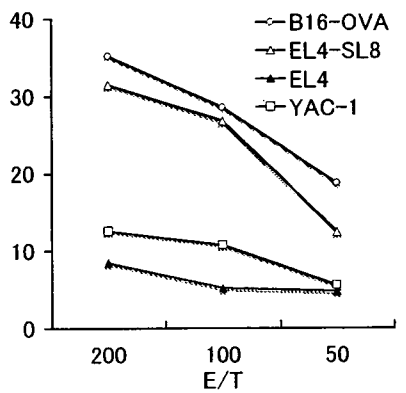
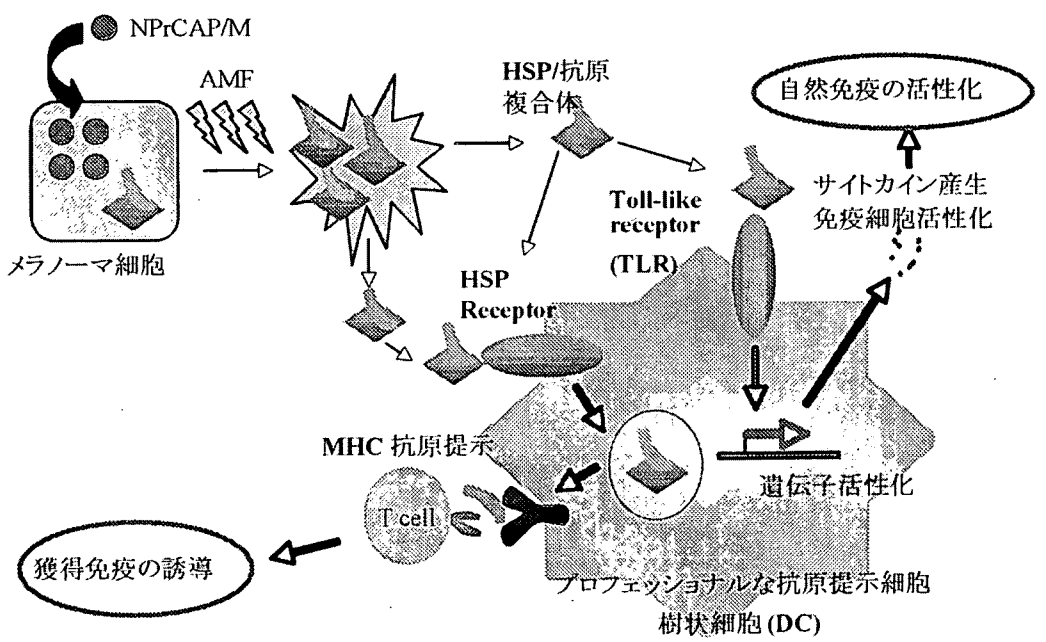


図 9　NPrCAP/M を用いた温熱免疫療法のメカニズム



D. 考察

我々はメラノーマ細胞に選択的にとりこまれ細胞内発熱効果をもつ NPrCAP/M を用いて選択的にメラノーマ細胞を温熱する Chemo-Thermo-Immuno (CTI) Therapy の確立を目指している。NPrCAP はメラノサイトおよびメラノーマが特異的に発現しているチロシナーゼの強力な基質となるため、メラノーマへの targeting をはかり、magnetite の表面にこれを結合させることで、細胞内での加温が遂行可能となる。すなわち本治療法では NPrCAP/M に磁場照射を加えることにより、メラノーマ選択的な温熱療法が可能である。実際、in vivo においても NPrCAP/M と磁場照射を３回施行することで、5mm 大の B16-OVA メラノーマ細胞はほぼ完全に退縮した。これらの腫瘍退縮をみたマウスに再度 B16-OVA を接種すると、完全に拒絶した。一方、同系のマウス肺癌細胞 3LL を接種したマウスでは、このような腫瘍拒絶は認められなかった。これらの事実は、NPrCAP/M による温熱治療によって腫瘍退縮を得たマウスでは、メラノーマ特異的な抗腫瘍免疫が誘導されていることが示唆する。また NPrCAP 投与で腫瘍が退縮したマウスでは、B16-OVA に対する高い細胞傷害活性を有する細胞傷害性 T 細胞(CTL)の誘導を認めた。この CTL は OVA 由来の $H\text{-}2K^b$ に提示される SL8 ペプチド(SIINFEKL)をパルスした標的細胞を傷害した。このことは NPrCAP/M と温熱によるメラノーマ細胞の necrosis は“in situ” vaccination として抗腫瘍免疫応答を誘導する、いわゆる immunogenic cell death であると考えられる。図９に示すように、この免疫応答は HSP の up-regulation、特に Hsp72 が重要な役割を果たしていることを示した。この Hsp72 はメラノーマ細胞内で抗原ペプチドを結合しており、細胞の壊死により細胞外に放出される、この Hsp72-抗原ペプチド複合体が樹状細胞などの抗原提示細胞に取り込まれ、さらに抗原ペプチドがその MHC class Ⅰ分子に提示される、すなわちクロスプレゼンテーションされることで、特異的 CTL を誘導・活性化することを示した。このように CTI 療法により、全身性の抗腫瘍免疫応答を誘導することが可能であり、遠隔転移したメラノーマに対しても治療効果が期待できることを示している。またメラノーマの征圧のためにはこの抗腫瘍免疫効果を生涯にわたり持続させることが肝要である。CTI 療法で一旦誘導された免疫応答に対するブースター免疫の開発が今後の重要な課題である。

E. 結論

我々が開発したチロシンのアナログである NPrCAP と AMF によるメラノーマ標的温熱免疫療法はは、すでに樹立したマウスメラノーマ腫瘍に対して著明な腫瘍増殖抑制効果を示した。完全な腫瘍退縮を得たマウスにおいては、$CD8^+$ T 細胞をエフェクター細胞とする腫瘍特異的な免疫応答が誘導されていることを示した。この事実は NPrCAP/M をメラノーマに取り込ませたのち、温熱をメラノーマに与えることにより、免疫賦活性の細胞死を介する免疫応答を惹起し、転移巣の退縮を期待できるものである。今後、全身性の抗腫瘍免疫の維持をはかるための適切なブースター免疫を開発することも重要な課題である。また深部の再発・転移にも有用な治療法の開発も念頭に置く必要がある。

G. 研究発表

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H. 知的財産権の出願・登録状況

1. 特許取得

1．田村保明, 大浦　淳、鳥越俊彦、佐藤昇志：アレルギー性疾患の予防・治療薬。特願 2007-050811, 平成１９年２月２８日

2. 実用新案登録

なし。

3. その他

なし。

Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
なし							

雑誌

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Ito A, Fujioka M, Yoshida T, Wakamatsu K, Ito S, Yamashita T, Jimbow K, Honda H	4-S-Cysteaminylphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma	Cancer Sci	98(3)	424-430	2007
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Okochi M, Hayashi H, Ito A,	Identification of HLA-A24-restricted	J Biosci Bioeng	in press	in press	in press

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IV. 研究成果の刊行物・別冊

Melanoma-Targeted Thermotherapy Using N-propionyl-4-S-cysteaminylphenol-Magnetite Nanoparticles Elicits CTL Response Via HSP-Peptide Complex Release.

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Departments of [1]Dermatology and [2] Pathology, Sapporo Medical University, Sapporo; [3]Department of Chemical Engineering, Faculty of Engineering, Kyusyu University, Fukuoka; [4]Department of Biotechnology, Nagoya University, Nagoya and [5]Department of Chemistry, Fujita Health University, Toyoake, Japan

Running title: Melanoma-targeted hyperthermia elicits CTL via HSP induction

Key words: melanoma, hyperthermia, tyrosinase, heat shock protein, cytotoxic T cell

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Abstract
We have demonstrated that a tyrosinase substrate, N-propionyl-4-S-cysteaminylphenol (NPrCAP) is specifically taken-up by melanoma cells. By taking advantage of this unique chemical agent, we have established melanoma-targeting intracellular hyperthermia by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/M) and exposing alternating magnetic field (AMF). This treatment causes cytotoxic reaction, leading to elicitation of antitumor immune response, which was proved by tumor re-challenge test and CTL induction. We found the level of heat shock protein 72 (Hsp72) to be increased in the cell lysate and culture supernatant after intracellular hyperthermia. $CD8^+$ T cells responding to dendritic cells, which were loaded with cell lysate derived from intracellular hyperthermia were also detected. Thus, it is suggested that the *in vivo* rejection of re-challenged melanoma after hyperthermia derives from the release of HSP-peptide complex from degraded tumor cells, which can elicit specific tumor immunity against melanoma cells.

Introduction
Melanoma has been increasing in incidence leading to a rise in morbidity and mortality in recent decades. Metastatic melanoma is extremely difficult to cure and continues to have a poor prognosis. Only 12% with metastatic melanoma survive for five years (1). The reason for this poor prognosis is the lack of effective conventional therapies. Various types of therapies such as immunotherapy, chemotherapy and biologic therapy have been studied in melanoma management. However, a very modest effect was recorded in advanced malignant melanoma. Therefore, there is an emerging need for innovative therapies for the control of advanced melanoma.

It has been reported that the intracellular hyperthermia using magnetic nanoparticles is effective for treating certain types of cancer in not only primary but also metastatic lesions (2, 3). Incorporated magnetic nanoparticles generate heat within the cells after exposure to the alternating magnetic field (AMF) due to hysteresis loss (4). One of us showed that hyperthermic treatment using magnetite cationic liposome induces antitumor immunity by enhancement of heat shock protein (HSP) expression (3, 5-7). HSPs have been demonstrated to chaperone antigen peptides derived from the cells (8). Immunization with tumor-derived HSP-peptide complex is able to elicit tumor-specific T cell response (9-12). In addition, HSP-peptides complexes would be released into the extracellular milieu as a consequence of pathological cell death including hyperthermic treatment. Tumor-derived HSP released into the extracellular milieu has been shown to act simultaneously as an antigen source due to its ability to chaperone peptides and as a maturation signal for dendritic cells (DCs), thereby inducing DCs to cross-present antigens to $CD8^+$ T-cells, resulting in tumor-specific cytotoxic T cell (CTL) induction (13, 14). Based on these findings, it was suggested that this hyperthermic treatment elicited "*in situ* vaccination" with Hsp70-peptide complex via necrotic tumor cell death (7). However, this treatment has the disadvantage of causing damage to not only cancer cells but also to non-cancerous tissue due to non-specific incorporation of magnetite cationic liposome. Therefore, it is desirable to selectively deliver the magnetite particle to tumor cells in order to accomplish intracellular hyperthermia. If this tumor cell specific-hyperthermia could induce systemic antitumor immunity through up-regulation of HSPs, this would enable us to control distant metastasis, leading to an improvement in prognosis.

We have previously shown that the sulfur-amine analog of tyrosine, cysteaminyl phenol (CAP) and its N-acetyl or propionyl derivatives (NPrCAP) are good substrates of melanoma and are able to cause selective cytotoxicity against melanocytes and melanoma cells. They are good candidates for developing anti-melanoma chemotherapy because melanogenesis is inherently toxic and expressed uniquely in melanocytic cells(15-17). We introduced the use of phenolic thioester amines of tyrosine to target melanoma cells based on the idea that the incorporation of sulfur

would render the phenols more cytotoxic by increased lipophilicity leading to increased uptake by cells, thus providing more tyrosinase substrates available to cells. In fact, we have shown that NPrCAP was selectively incorporated by melanocytes and melanoma cells and thereafter showed specific cytocidal effects (18). Therefore, we synthesized magnetite nanoparticles conjugated with NPrCAP (NPrCAP/M). NPrCAP/M is specifically targeted to melanoma cells, and internalized and aggregated within their cell cytoplasm. Furthermore, we have observed that B16-OVA cells, which were subjected to intracellular hyperthermia using NPrCAP/M with AMF exposure, were brought to necrotic cell death (Sato M, *et al.* manuscript submitted). Here we studied how and what extent intracellular hyperthermic treatment using NPrCAP/M with AMF exposure is feasible against B16-OVA melanoma cells. We found that this novel melanoma-targeting intracellular hyperthermia induces anti-melanoma immunity through up-regulation of intracellular and extracellular HSPs-peptide complex derived from melanoma cells.

Materials and Methods

Mice and cells.

Female C57BL/6 mice were obtained from Hokudo (Sapporo, Japan) and used at 4 to 6 week of age. B16-OVA is a B16F1 cells stably transfected with chicken ovalbumin (OVA) cDNA (kindly provided from Dr. Y. Nishimura, Kumamoto University, Kumamoto, Japan). B16-OVA was cultured in RPMI supplemented 10% fetal bovine serum and 250 μg/ml of hygromycin B at 37℃ in a 5% CO_2 atmosphere. B3Z is a $CD8^+$ T cell hybridoma that expresses LacZ in response to activation of T cell receptors specific for the SIINFEKL peptide (SL8; OVA-immunodominant peptide) in the context of H-$2K^b$ MHC class I molecules (kindly provided from Dr. N. Shastri, University of California, Berkeley, CA). When activated, this hybridoma expresses LacZ, allowing a simple colorimetric measurement of Ag-specific T cell stimulation. 3LL lung carcinoma cells were cultured in DMEM supplemented with 10% heat-inactivated FCS. EL4 lymphoma cells, YAC-1 cells and B3Z cells were cultured in complete RPMI supplemented with 10% FCS at 37℃ in a 5% CO_2 atmosphere. Bone marrow-derived DCs were generated from the femurs and tibiae of C57BL/6. The bone marrow was flushed out, and the leukocytes were obtained and cultured in complete RPMI1640 with 10% FCS and 20 ng/ml GM-CSF (Endogen, Inc., Woburn, MA) for 5 days. On day 3, fresh medium with GM-CSF was added to the plates for the day 5 cultures.

Chemicals

4-S-cysteaminylphenol (4-S-CAP) was prepared as described by Padgette *et al*(19). N-succinimidyl-3-[2-pyridyldithio] propionate (SPDP) was obtained from Molecular Biosciences Inc. (Boulder, CO). All other chemicals were of analytical grade.

Synthesis of N-(1-mercaptopropionyl)-4-S-cysteaminylphenol (NPrCAP-SH)

A mixture of 1.81 g of 4-S-CAP (10.7 mmol) and 4.13 g of SPDP (13.2 mmol) in 5 ml of pyridine was stirred for 2 hrs at room temperature. The yellow solution was evaporated under a vacuum pump. The residue was purified by a silica gel column chromatography (ethyl acetate : n-hexane; 2:1 v/v as eluant) to give a disulphide (3.70 g; 94%). To a stirred solution of the disulphide (3.70 g; 10.3 mmol) in 5 ml of methanol, 4.29 g of dithiothreitol (DTT, 27.5 mmol) was added at room temperature. After 2 hrs the mixture was evaporated and the oily residue was purified by a silica gel column chromatography (ethyl acetate : n-hexane; 2 : 1 v/v) to give 2.19 g of NPrCAP-SH (80%) as a colorless crystal after re-crystallization (ethyl acetate – ether). The resulting material was subjected to the liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis using an electronspray ionization/ion trap mass spectrometer (LCQ Deca XP, Thermoelectron, Tokyo, Japan). The analysis was carried out directly by the MS/MS at positive charge; $[M+H]^+$: *m/z* 258, 164, 153, 132, 125. ^{1}H-NMR was measured at 400 MHz (CD_3COCD_3): 2.45 ppm (2H, t, J = 0.68 Hz), 2.71 ppm (2H, m), 2.88 ppm (2H, t, J = 1.1 Hz), 3.32 ppm (2H, m), 6.68 ppm (2H, d, J = 0.80 Hz), 7.31 ppm (2H, d, J = 0.80 Hz).

Preparation of NPrCAP/M

Magnetite nanoparticles (Fe_3O_4; average particle size, 10 nm) were kindly provided by Toda Kogyo (Hiroshima, Japan). Iron concentration of magnetite nanoparticles was measured using the potassium thiocyanate method. First, in order to prepare the aminosilane-coated magnetite nanoparticles, 10 ml of 40 mg/ml magnetite nanoaprticles and 0.1 ml of 3-aminoprpyltriethoxysilane (Tokyo Chemical Industry, Tokyo, Japan) were mixed and incubated for one hr with stirring at room temperature. The resultant magnetic suspension was then washed three times with water by centrifuging at 2500 rpm for 2 min. Next, in order to conjugate maleimide cross-linkers, the magnetite suspension thus prepared was mixed with 200 ml of 10 mg/ml N-[γ-maleimidobutyryloxy] sulfosuccinimide ester (sulfo-GMBS, Pierce, Rockford, IL) and incubated in PBS for 30min with shaking at room temperature. The resultant magnetite suspension was washed three times with water by centrifugation at 2500 rpm for 2 min.

Then 0.5 ml of 50 mg/ml NPrCAP-SH was added to 10 ml of magnetite suspension (40 mg/ml) and the mixture was stirred for 30 min at room temperature. After leaving the suspension for two hrs at room temperature the suspension was washed twice with water by centrifugation at 3000 rpm for one min. The resultant NPrCAP/M was resuspended with 10 ml of water.

Analysis of incorporated NPrCAP in magnetite nanoparticles

The degree of incorporation of NPrCAP-SH to magnetite was determined by the hydrolysis with 6M HCl followed by HPLC analysis of 4-S-CAP produced. Briefly the amount of 4-S-CAP produced by the hydrolysis of NPrCAP/M

with 6M HCl 110°C for 1.5 hrs was measured by HPLC which consisted of a Jasco PU-980 intelligent liquid chromatogram with a Jasco 851-AS intelligent autosampler (JASCO, Tokyo, Japan), a Jasco 875-UV/VIS detector, and Shiseido C18 reverse-phase column (Capcell pak C18, 4.6 x 250 mm; 5 μm particle size). The UV detector was set at 250 nm. The mobile phase used was methanol : H_2O water : 1.0 M $HClO_4$, 10 : 90 : 1.5 by vol. The analyses were performed at 50°C at a flow rate of 0.7 ml/min. The results indicated that the degree of incorporation of NPrCAP-SH to magnetite was 405 nmol/mg magnetite. Iron concentration of magnetite nanoparticles was measured using the potassium thiocyanate method(20).

Antibodies.

Anti-H-2K^b/D^b mAb (clone 28-8-6) and anti-H-2K^d mAb (clone SF1-1.1) were purchased from BDPharMingen. For depletion of HSPs from cell lysate, anti-Hsp72/Hsc73 mAb, anti-Hsp90α polyclonal antibody, anti-Hsp90 mAb and anti-KDEL mAb were used. Anti-Hsp72/Hsc73 mAb and anti-Hsp90mAb were used for western blotting. These antibodies were obtained from StressGen Biotechnologies (British Columbia, Canada). Mouse IgG and Rabbit IgG were purchased from IBL (Takasaki, Japan).

Transplantation of tumor cells and intracellular hyperthermia.

B16-OVA cells ($1X10^6$) were subcutaneously transplanted into the right flank of C57BL/6 mice on day 0. NPrCAP/M nanoparticles (100 μl) were injected into the tumor on day 7, 9, 11, and 13. A magnetic field was created using a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Dai-ichi High Frequency, Tokyo) (4). The magnetic field frequency and intensity were 118 KHz and 30.6 KA/m (386 Oe), respectively. 24 hrs after injection, mice were subjected to AMF exposure to heat the tumor at 43°C for 30 min. Tumor growth was recorded once every two days. The cured mice were then re-challenged with a subcutaneous injection of B16-OVA cells ($1X10^6$) or irrelevant 3LL lung carcinoma cells ($1X10^6$) on the left flank. Size was determined by the following formula; tumor volume = 0.5 X (length X width^2), where length and width are measured in millimeters.

Histopathology of tumor sections.

Subcutaneous B16-OVA tumors were harvested and fixed in 10% Formalin in PBS, then paraffin embedded and sectioned. H&E-stained sections were prepared for analysis of therapeutic effect and gross infiltrate.

In vitro cytotoxicity assay.

After mice were treated by intracellular hyperthermia as described above, spleens were harvested on day 28, then $5X10^6$ spleen cells were re-stimulated *in vitro* with irradiated B16-OVA cells in 2 ml of complete medium (RPMI medium supplemented with 50 μM of β-mercaptethanol (GIBCO BRL) and 10% FCS) for 5 days. Cytotoxic activity of the effector cells against target cells (B16-OVA, EL4, EL4-SL8 (EL4 pulsed with SL8 peptide) and YAC-1) was determined by standard ^{51}Cr release assay. To determine the MHC class I restriction in this cytotoxicity assay, indicated concentration of mAb against H-2K^b/D^b or H-2K^d was added to each well.

Quantification of HSPs.

Cultured B16-OVA cells were exposed to NPrCAP/M for 30 min and irradiated by alternating magnetic field (AMF) to heat them at 43°C. After NPrCAP/M exposure with or without AMF irradiation, cells ($1x10^6$) were cultured in 1 ml of 10% RPMI for 72 hrs. Culture supernatant was collected at 12, 24, and 48 hrs or cells escaped from cell death, were lysed at 72 hrs after intracellular hyperthermia by freezing and thawing and centrifuged at 3500 rpm for 5 min. The expression of Hsp72/Hsc73 and Hsp90 was determined by western blotting with an anti-Hsp72/Hsc73 mAb or anti-Hsp90 mAb. HSP in the lysate or culture supernatant was quantified by Hsp90α ELISA kit and Hsp70 ELISA kit (StressGen), which can detect and quantify Hsp90α and inducible Hsp72, respectively.

OVA re-presentation and T cell activation assay.

Response of B3Z T cell hybridoma was assayed by the measurement of LacZ activity using chlorophenol red-β-D-galactopyranoside (CPRG) (Sigma). Cultured B16-OVA cells were exposed to NPrCAP/M for 30 min and irradiated by AMF to heat them at 43°C. After NPrCAP/M exposure with or without AMF irradiation, cells were cultured for 72 hrs and $1x10^7$ of cells in 1ml of 10% RPMI medium were lysed by three cycles of freezing and thawing and centrifuged at 3500 rpm for 5min. DCs ($1x10^5$) derived from bone marrow of C57BL/6 mice were pulsed with the cell lysate (100 μl) and incubated with $1x10^5$ B3Z T cell hybridoma. After overnight incubation, LacZ activity was measured by addition of 100 μl of CPRG solution. The absorbance was measured at 595nm after 4-hr-incubation at 37°C.

In vitro depletion assay.

Cultured B16-OVA cells were treated and lysed as described above. The cell lysate (100 μl) was incubated with antibodies (5 μg each) against Hsp90, Hsp72/Hsc73, and KDEL. The mixture was added to 10 μl of protein A-Sepharose beads (50% slurry, Pharmacia) and the suspension was rotated at RT for 1hr. Then, the suspension was spun at 10,000 rpm for 1 min. After removal of the beads, the supernatant was used for OVA re-presentation and T cell activation assay as described above. Mouse IgG (15 μg) was used for experimental control. Depletion was

assessed by immunoblotting with anti-Hsp90, Hsp72/Hsc73, or KDEL antibodies.

In vivo representation assay and induction of peptide-specific CTL.

C57BL/6 mice were immunized in their footpads with immunoprecipitates (in 50 μl PBS) made with a mixture of antibody against Hsp72/73, Hsp90 and KDEL, or made with control mouse IgG from the tumor lysate. After 5 hrs of immunization, popliteal nodes were removed and DCs were isolated using CD11c MACS beads (Miltenyi Biotec). Then, B3Z cells ($1x10^5$) were added to the DC culture ($1x10^5$) in 96-well flat-bottom plates and incubated at 37℃. Twenty-four hrs after incubation, absorbance at 595nm was measured. B3Z cells recognize the $OVA_{257-264}$ peptide presented by H-$2K^b$ and express lacZ upon activation. For in vivo CTL induction, C57BL/6 mice were immunized s.c. with bone marrow-derived DCs ($1X10^6$) pulsed with immunoprecipitates made with a mixture of antibody against Hsp72/73, Hsp90 and KDEL, or made with control mouse IgG on days 0 and 7. Seven days after the second immunization, splenocytes from immunized mice were cultured in the presence of 1 μg/ml SL8 peptide at $5x10^6$ cells/ml for 5 days. On days 5, cells were harvested for standard 4-hr chromium release assay.

Statistical analyses.

We determined statistical significance using the Kruskal-Wallis one-way analysis. In all experiments, differences were considered statistically significant at $P < 0.05$.

Results

Antitumor effect of intracellular hyperthermia using NPrCAP/M with AMF exposure and its ability to induce anti tumor immunity.

We examined the efficacy of combination therapy of NPrCAP/M and hyperthermia against murine melanoma B16-OVA tumor growth and its impact on antitumor effect. Mice were transplanted with B16-OVA melanoma cells and treated with NPrCAP/M injection followed by hyperthermia or NPrCAP injection alone as described in the materials and methods. Histopathological examination of the tumors without treatment showed that inflammatory infiltrates were poorly detected (Fig. 1A-a and 1A-b). In contrast, treatment with combination of NPrCAP/M injection and hyperthermia induced apparent tumor destruction and necrosis with deposit of NPrCAP/M particles (Fig. 1A-c and 1A-d). In addition, a dense inflammatory infiltrate was observed around the residual tumor cells (Fig. 1A-e). We have shown that this infiltrate included both $CD8^+$ T cells and $CD4^+$ T cells (Takada, *et al.* manuscript submitted). Tumor volume in the group treated by combination of NPrCAP/M injection and hyperthermia was significantly reduced compared with the non-treated control group ($P = 0.0025$) and the group of NPrCAP alone ($P = 0.023$) (Fig.1B).. Six out of ten mice in the group treated by NPrCAP/M injection and hyperthermia were cured. To examine whether cured mice developed the antitumor immune responses, these mice were re-challenged with live B16-OVA melanoma cells or irrelevant mouse lung carcinoma 3LL two weeks after NPrCAP/M and hyperthermic treatment. As a result, all cured mice rejected a re-challenge of live B16-OVA melanoma cells, but not 3LL lung carcinoma cells (Fig. 1C). These data indicated that intracellular hyperthermia using NPrCAP/M with AMF exposure induced specific antitumor immunity.

Induction of tumor-specific CTL by intracellular hyperthermia.

To analyze the mechanism for the generation of antitumor immunity by NPrCAP/M and hyperthermia, we examined CTL induction in mice after intracellular hyperthermia. Spleen cells of mice after hyperthermia showed high cytotoxicity against B16-OVA melanoma cells compared to EL4 lymphoma and YAC-1 cells. In addition, spleen cells also showed high cytotoxicity against EL4 pulsed with SL8 peptide derived from OVA protein (Fig. 2A). We performed blocking experiments using anti-H-2-specific mAbs. Cytotoxicity of the CTLs was specifically inhibited dose-dependently by an anti-H-$2K^b$/D^b mAb but not by an anti-H-$2K^d$ mAb (Fig. 2B), thus demonstrating that the cytolytic activity of the CTLs induced in vitro was antigen-specific and MHC class I-restricted. These results suggest that intracellular hyperthermia using NPrCAP/M can elicit specific tumor immunity by inducing CTL against B16-OVA melanoma cells.

Enhanced expression of Hsp72 in B16-OVA melanoma cell after intracellular hyperthermia.

We examined the expression of HSPs in tumor cells treated with NPrCAP with AMF exposure in vitro by western blotting and ELISA. Protein level of Hsp72 but not Hsc73 or Hsp90 was increased at 48 hrs after hyperthermia (Fig. 3A). Similarly, the concentration of Hsp72 in cell lysate resulted in a 3-fold increase at 72 hrs after hyperthermia, compared to cells without treatment (Fig. 3B). However, the concentration of Hsp90 did not change (Fig. 3C). Treatment with NPrCAP alone decreased the level of intracellular Hsp72 and Hsp90. We are currently investigating the underlying mechanism. From the results obtained, we hypothesized that hyperthermia using NPrCAP/M induces tumor immunity through up-regulation of Hsp72.

Intracellular hyperthermia using NPrCAP/M with AMF exposure results in the release of HSPs into the extracellular milieu.

It has been demonstrated that once cancer cells become necrotic, several HSPs, above all, Hsp72 and Hsp90 are released from cells and might act as a danger signal, subsequently eliciting cell-specific immune responses. We therefore examined whether Hsp72 and Hsp90 would be released from necrotic melanoma cells after intracellular hyperthermia in vitro. Culture supernatants from B16-OVA were collected at 12, 24, and 48 hrs after intracellular hyperthermia and the quantity of Hsp72 and Hsp90 was evaluated using ELISA. Although Hsp72 and Hsp90 were

detected at 48 hrs after hyperthermia, concentration of extracellular Hsp72 was a 45-folds higher than that of Hsp90 (Fig. 3D). These in vitro results suggested that treatment of B16-OVA melanoma with intracellular hyperthermia would release HSPs, in particular Hsp72, into extracellular milieu in vivo and these extracellular HSPs might play an important role in inducing antitumor immunity.

$CD8^+$ T cell response against DCs pulsed with B16-OVA melanoma cell lysate after intracellular hyperthermia.

To analyze the mechanism of tumor specific CTL induction, we examined B3Z $CD8^+$ T cell response against DC pulsed with supernatant from B16-OVA culture after intracellular hyperthermia. However, only a very modest response was observed (data not shown). One of the reasons to this modest response may be due to the degradation of peptide chaperoned by HSPs by protease in the culture medium. We therefore decided to use melanoma cell lysate after hyperthermia. NPrCAP loaded B16-OVA melanoma cells were subjected to AMF irradiation and lysed by 3 cycles of freezing and thawing. DCs derived from mouse bone marrow were pulsed with the lysate for 2 hrs and then cultured with B3Z $CD8^+$ T cell hybridoma. B3Z response against DC pulsed with B16-OVA melanoma cell lysate increased after intracellular hyperthermia using NPrCAP/M, compared with non-heated cells and cells loaded NPrCAP/M without AMF exposure (Fig.4A). These data demonstrated that loading DCs with lysate derived from melanoma cells treated with hyperthermia enhanced the cross-presentation of B16-OVA-specific antigen peptide.

Effects of immunodepletion of HSPs on $CD8^+$ T cell response.

Next, we investigated the underlying mechanism responsible for the enhancement of cross-presentation in the case of intracellular hyperthermia. Hyperthermia has long been shown to induce the expression of HSPs, which have been shown to chaperone tumor-associated antigen peptides. To investigate the role of HSPs in intracellular hyperthermia-induced $CD8^+$ T cell response, we depleted HSPs from lysate using anti-HSP antibody and measured $CD8^+$ T cell response against DCs pulsed with the immunodepleted lysate. Depletion of major HSPs (Hsp72/Hsc73, Hsp90, and ER-resident HSPs) from NPrCAP/M and hyperthermic treated B16-OVA cell lysate caused a loss of 59% of initial B3Z response ($P = 0.0001$, versus depletion with control Ig) (Fig 4B), whereas depletion with control Ig did not show any effect. Importantly, depletion of Hsp72/Hsc73 exhibited a 44% reduction of activity and it was best decrease in response in the HSP depletion assay. The inhibition rate was statistically significant compared with the depletion with control Ig ($P = 0.001$). Depletion of Hsp90 or ER resident HSPs caused a loss of 25% ($P = 0.0857$) or 31% ($P = 0.0034$) of the initial activity, respectively. Immunoblots showed that depletion of each HSPs was complete (Fig. 4C). These results suggested that Hsp72/Hsc73, Hsp90, and ER-resident HSPs were involved in induction of CTL response at various extents. In addition, our data demonstrated that these HSPs chaperoned antigenic peptides and extracellular HSP-peptide complexes were cross-presented by DCs, followed by specific CTL activation. Notably, Hsp72/Hsc73 was largely responsible for the observed T cell response. As shown in Fig. 3, these data were consistent with the enhanced expression of Hsp72 within the melanoma cells. Moreover, as Hsp72 was shown to act as an immune adjuvant for cross-presentation by DCs, hyperthermia-induced Hsp72 might elicit cross-presentation-competent DC. Thus, DCs loaded with intracellular hyperthermia-treated melanoma cell lysate is more efficient than DCs loaded with untreated melanoma cell lysate in cross-presentation to CTLs.

Tumor-derived HSPs-peptide complexes are cross-presented by DCs derived from regional lymphnodes.

We examined whether tumor-derived HSP-peptide complex is cross-presented by $CD11c^+$ DCs in vivo. To test this, we immunized mice with immunoprecipitates made with a mixture of antibody against Hsp72/Hsc73, Hsp90 and KDEL from hyperthermia treated B16-OVA tumor lysate as tumor-derived HSPs. B3Z response against regional lymph node-derived DCs of HSP-immunized mice was evident. In contrast, DCs derived from mice immunized with immunoprecipitates made with control Ig from the same tumor lysate could not stimulate B3Z response (Fig 5A). We therefore conclude that intracellular hyperthermia using NPrCAP/M with AMF exposure promotes OVA-derived peptide presentation on DCs through up-regulation and release of HSP-peptide complex.

Immunization of tumor-derived HSPs-peptide complex elicits peptide-specific CTLs in vivo.

We further tested whether mice immunized with immunoprecipitates made with a mixture of anti-HSP antibody from B16-OVA tumor lysate, which were subjected to the intracellular hyperthermia, could induce tumor peptide-specific CTLs. As shown in Fig. 5B, immunization with the immunoprecipitates containing B16-OVA derived HSPs induced a strong OVA-specific CTL response. No CTL activity was observed in control mice (Fig. 5C). These results further confirmed that tumor-derived HSPs chaperoned antigenic peptide(s)

Discussion

In this study, we showed that intracellular hyperthermia of melanoma cells using NPrCAP/M with AMF exposure enhanced their immunogenicity, resulting in augmentation of cross-presentation of melanoma antigen via up-regulation of intracellular and extracellular HSP-peptide complex. Our study had three main findings: (a) treatment of mice with hyperthermia using NPrCAP/M elicits B16-OVA specific CTL response, which is capable of killing cells pulsed with OVA-immunodominant peptide in vitro, (b) peptide specific $CD8^+$ T cell response against DCs increased when DCs were pulsed with B16-OVA cell lysate after hyperthermia using NPrCAP/M in vitro by cross-presentation, (c) The enhanced cross-presentation was mainly dependent on the up-regulation of Hsp72. These results provide evidence that hyperthermia using NPrCAP/M can elicit specific tumor immunity against melanoma cells through the up-regulation and release of HSP-peptide complex from necrotic tumor cells. Namely, the most straightforward explanation for the enhanced cross-presentation is that augmentation of HSPs expression allows the association and transfer of melanoma antigen peptides onto DCs MHC class I molecules (Fig. 6).

It has been believed that enhanced expression of intracellular HSPs by hyperthermia plays an important role in the

induction of antitumor immunity (5, 21). However, the mechanism of induction of HSPs within the tumor cells is controverted. In fact, it has been shown that hyperthermia induces intracellular HSP, resulting in the inhibition of apoptosis (22). In contrast, over-expression of HSPs, particularly Hsp72, causes increased tumor immunogenicity due to augmentation of the chaperoning ability of antigenic peptide, thereby augmenting the presentation of antigenic peptide in the context of MHC class I molecules (23, 24). However, in order to prime tumor specific immunity, it is necessary that tumor antigen should be presented in the context of MHC class I in conjunction with co-stimulation signal through co-stimulatory molecules such as B7.1 and B7.2 by professional antigen presenting cells such as DCs. DCs have the unique capacity to take up, process, and present exogenous antigens in association with MHC class I molecules. This process is termed cross-presentation and the resulting $CD8^+$ T cell priming is referred to as cross-priming. It has been demonstrated that some exogenous antigens such as HSPs and particulate protein antigens (25) gain access to the MHC class I processing pathway and initiate CTL responses. This exogenous pathway is important for the development of $CD8^+$ CTL responses against tumors and infectious pathogens that do not have access to the classical MHC class I pathway. Administration of antigenic peptides in the context of purified HSPs induces potent $CD8^+$ T-cell responses, indicating that HSP-peptide complexes can access the MHC class I endogenous antigen presentation pathway (12, 26, 27). Thus, shuttling exogenous peptides into the endogenous pathway might be a specialized function of HSPs. However, the precise mechanism of HSP-mediated cross-presentation remains to be elucidated. Recently, we have demonstrated that exogenous Hsp90-antigen peptide complex is efficiently cross-presented to $CD8^+$ T cells and elicit antigen-specific tumor immunity via an endosomal pathway by DCs (28).

Here, we show that the HSPs-antigen peptide complex released from melanoma cells treated with intracellular hyperthermia is taken-up by DCs and cross-presented HSP-chaperoned peptide in the context of MHC class I molecules. Our data suggested that Hsp72/Hsc73, Hsp90, and ER-resident HSPs participated in the induction of $CD8^+$ T cell response. In particular, among HSPs, Hsp72 was largely responsible for the augmented antigen presentation to $CD8^+$ T cells. As Hsp72 is known to up-regulate in response to hyperthermia or heat shock treatment, newly synthesized Hsp72 has a chance to bind to the heat-denatured melanoma-associated antigen. When melanoma cells suffered necrotic cell death by repeated intracellular hyperthermia, large amount of released Hsp72 could be taken-up by DCs, subsequently Hsp72-chaperoned antigen peptide is cross-presented to $CD8^+$ T cells.

Recently, a number of cellular receptors for several kinds of HSPs have been described, including CD91 (29), CD40 (30), TLR2/4 (31), LOX-1 (32), and SR-A (33). CD40 and LOX-1 are expressed on the cell surface of APCs including DCs and are known to be Hsp72 receptors for efficient antigen cross-presentation. The interaction of Hsp72-peptide complex with these receptors facilitates receptor-dependent endocytosis, processing of the chaperoned peptides, and re-presentation of peptide in the context of MHC class I molecules. Therefore, it was considered that released Hsp72-peptide complex induced antigen-specific tumor immunity very efficiently by receptor-mediated endocytosis, followed by cross-presentation. In addition, we demonstrated that other HSPs such as Hsp90 and ER-resident gp96, and BiP were also involved in the augmentation of cross-presentation, showing that these HSPs chaperoned melanoma antigenic peptide and were released by repeated hyperthermia-induced necrotic cell death.

Based on these findings, we are now performing a phase I/II study using our intracellular hyperthermia system for the treatment of advanced malignant melanoma. Our preliminary study indicated that one out of five cases cutaneous metastases regressed completely without major adverse effect. Intracellular hyperthermia using NPrCAP/M is a promising treatment for improvement of clinical effects, especially for patients with advanced metastatic melanomas, and even for prevention of recurrence and/or metastasis for early melanomas.

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LETTER TO THE EDITOR

High levels of melanin-related metabolites in plasma from pink-eyed dilution mice

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Dear Sir,

In mice, >120 genes are involved in the development, proliferation and differentiation of epidermal melanocytes, and more than half of those genes have been cloned and their functions elucidated (Bennett and Lamoreux, 2003). Mutations in many of these genes affect the quantity or quality of melanin produced that is usually measured by chemical determination of melanin content in cells or in hair. Whether mutations in specific genes differentially affect plasma levels of such melanin-related metabolites is not known. In this report, we reveal that pink-eyed dilution mice but not mice with mutations in the melanocortin-1 receptor (Mc1r) or the enzyme dopachrome tautomerase (Dct), exhibit extremely high plasma levels of these melanin-related metabolites.

Mammalian melanocytes produce two chemically distinct types of melanin pigments, the black to brown eumelanins and the yellow to reddish pheomelanins (Ito, 2003). Both eumelanin and pheomelanin are derived from the common precursor dopaquinone that is formed following the oxidation of tyrosine by tyrosinase. Dopaquinone is a highly reactive intermediate, and in the absence of thiol compounds it undergoes an intramolecular cyclization to give dopachrome through a redox reaction. However, intervention of this process with thiols such as cysteine gives rise exclusively to thiol adducts of dopa, termed cysteinyldopas, among which 5-*S*-cysteinyldopa (5-*S*-CD) is the major isomer. Dopachrome is spontaneously rearranged to give mostly 5,6-dihydroxyindole (DHI) with a trace of 5,6-dihydroxyindole-2-carboxylic acid (DHICA). However, the rearrangement of dopachrome to form DHICA is greatly accelerated by Dct, also termed tyrosinase-related protein-2, Tyrp2. Further oxidation of those indoles leads to the production of eumelanin, while cysteinyldopa adducts are oxidized to give pheomelanin. In melanocytes (and in hepatocytes), DHICA is *O*-methylated to give 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C) as a major isomer (Wakamatsu *et al.*, 1990).

Dct is the product of the *slaty/Dct* locus and is a member of the tyrosinase-related protein family. The *slaty* mutation changes an arginine to glutamine in the first copper binding domain of Dct, which reduces Dct function *c.* 80% compared with wild-type Dct in eye extracts. Moreover, Dct-mutant, immortalized melanocytes were recently found to have Dct activity at levels about one-third that of non-agouti black melanocytes (Costin *et al.*, 2005). In slaty melanocytes in primary culture, it has also been reported that the expression of Tyrp2/Dct was greatly reduced compared with melanocytes wild-type at that locus (Hirobe *et al.*, 2006). The *extension/Mc1r* gene at the *E* locus encodes Mc1r that is localized on the plasma membrane of melanocytes (García-Borrón *et al.*, 2005). Pigmentation is switched from the production of eumelanin to pheomelanin in recessive yellow (*e/e*) mice, which are homozygous for a loss-of-function mutation at the *extension* (*E*) locus (Hirobe *et al.*, 2007). The mouse *pink-eyed dilution* (*p*) locus greatly reduces the production of eumelanin in melanocytes and in the retinal pigment epithelium (Hirobe *et al.*, 2003). The product of the *p* gene is an integral membrane protein that localizes in melanosomes. The p protein seems to be involved in regulating the maturation of melanosomes and in the stabilization or trafficking of melanosomal membrane proteins. It has been proposed that the p protein controls the processing and transport of tyrosinase (Toyofuku *et al.*, 2002). Another theory is that the p protein mediates melanosomal pH (Ancans *et al.*, 2001). In this study, plasma levels of 6H5MI2C and 5-*S*-CD were analysed in mice with *slaty*, *recessive yellow* or *pink-eyed dilution* mutations, to determine whether the levels of those intermediates reflect the effects of those genic substitutions and to compare them with levels of melanin contents in hair.

Figure 1A summarizes the changes during development (0.5–7.5 days) in plasma levels of 6H5MI2C in

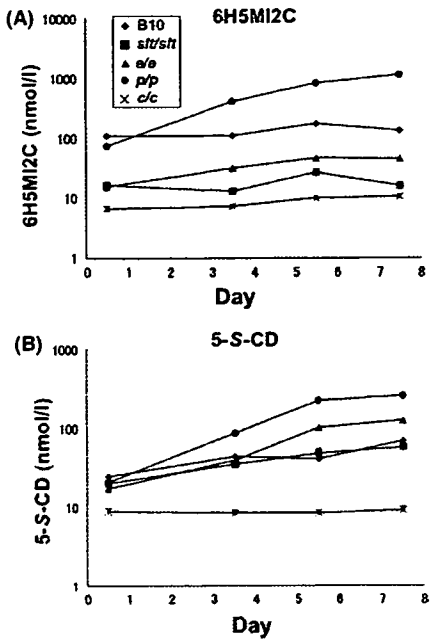

Figure 1 Changes in levels of (A) 6H5MI2C and (B) 5-*S*-CD in the plasma from 0.5-, 3.5-, 5.5- and 7.5-day-old C57BL/10JHir (black) mice and congenic *slt/slt* (slaty), *e/e* (recessive yellow), *p/p* (pink-eyed dilution), and *c/c* (albino) mice. Blood samples were collected from 0.5-, 3.5-, 5.5- and 7.5-day-old mice of all five strains from the carotid artery. Determination of 6H5MI2C in the plasma was performed using our previously reported method (Wakamatsu *et al.*, 1991) with minor modifications. Determination of 5-*S*-CD in the plasma was performed using HPLC with electrochemical detection, as previously reported by us (Wakamatsu and Ito, 1994) with a change of the plasma volume from 500 μl human serum to 10 μl mouse serum. Data are averages for three male and three female samples (one male and one female for *c/c* mice), each being taken from three to seven mice. There were no significant differences in plasma levels among male and female mice in each group, and thus the results present averages for all six samples. Standard errors of the mean (SEM) are smaller than symbols in most cases and are not shown.

C57BL/10JHir black mice (B10) and in congenic *slt/slt* (slaty), *e/e* (recessive yellow), *p/p* (pink-eyed dilution), and *c/c* (albino) mice. The black mice did not show any increase of 6H5MI2C during development with average levels of 144 nmol/l in their plasma. Similarly, levels of 6H5MI2C in plasma of *slt/slt* mice did not change significantly during the 7 day period, with an average level of 18 nmol/l which was sevenfold lower compared with black mice. Levels of 6H5MI2C in plasma of *e/e* mice showed a slight increase with age, from 16 nmol/l at 0.5 day to 45 nmol/l at 7.5 days. In contrast, levels of 6H5MI2C in plasma of *p/p* mice increased significantly with 74 and 1140 nmo/l at 0.5 and 7.5 days, respectively. The level of 6H5MI2C at 7.5 days in *p/p* mice was nine times greater than that in black mice. Albino mice gave a baseline level of 8.7 nmol/l on the average at all times examined. Figure 1B summarizes

Table 1. Plasma levels of 6H5MI2C and 5-*S*-CD in comparison with melanin contents in hair

	Plasma[a]		Hair[b]		
Phenotype	6H5MI2C (nmol/l)	5-*S*-CD (nmol/l)	PTCA[b] (ng/mg)	4-AHP[b] (ng/mg)	TM[b] (A_{500}/mg)
Black	134	70	1470	28	0.745
Slaty (*slt/slt*)	18	60	129	29	0.360
Recessive yellow (*e/e*)	70	127	9	2800	0.098
Pink-eyed dilution (*p/p*)	1140	258	70	91	0.051
Albino (*c/c*)	8.7	8.8	2	18	0.018

[a]Data are for 7.5-day-old mice.
[b]Most of the values are from unpublished studies, although similar data have been reported (Lamoreux *et al.*, 2001). Pyrrole-2,3,5-tricarboxylic acid (PTCA) is a specific degradation product from DHICA-derived eumelanin, while 4-amino-3-hydroxyphenylalanine (4-AHP) is from pheomelanin (Wakamatsu and Ito, 2003). Total melanin (TM) values were obtained by dissolving hair samples in Soluene-350 plus water (9:1, v/v) (Lamoreux *et al.*, 2001).

the changes during development (0.5–7.5 days) in plasma levels of 5-*S*-CD in C57BL/10JHir black mice (B10) and in congenic *slt/slt*, *e/e*, *p/p* and *c/c* mice. All four pigmented strains showed increased 5-*S*-CD levels with age. Black and *slt/slt* mice showed almost identical patterns of increases with 70 and 58 nmol/l at 7.5 days, respectively. Levels of 5-*S*-CD in *e/e* and in *p/p* mice at 7.5 days were 127 and 258 nmol/l, being 1.8 and 3.7 times higher than in black mice. Albino mice gave a baseline level of 8.8 nmol/l on the average at all time points.

Table 1 compares plasma levels of 6H5MI2C and 5-*S*-CD in 7.5 day newborn mice in comparison with melanin contents in hair. Mice mutant at the *slaty* locus (*slt/slt*) showed sevenfold lower levels of 6H5MI2C compared with black mice. This result is consistent with the greatly decreased activities of Dct in *slaty* mice (Costin *et al.*, 2005; Hirobe *et al.*, 2006) that lead to the production of DHI-rich and DHICA-poor eumelanin (Ito, 2003). The ratio of DHICA to DHI is known to correlate with the ratio of pyrrole-2,3,5-tricarboxylic acid (PTCA) to total melanin (TM) (Ozeki *et al.*, 1997). As shown in Table 1, *slaty* mice have a greatly reduced PTCA/TM ratio (360 ng/A_{500}) compared with black mice (1980 ng/A_{500}).

Recessive yellow (*e/e*) mice produce pheomelanic pigment in follicular melanocytes in vivo (Table 1). Those mice also show increases in the production of high levels of pheomelanin in the epidermis and dermis as the developmental age advances (Hirobe *et al.*, 2007). In the present study, plasma levels of 5-*S*-CD showed similar developmental increases. Moreover, the ratio of 5-*S*-CD/6H5MI2C levels in *e/e* mice was 2.8 at 7.5 days, while the ratio in black mice was 0.52, reflecting the type of pigmentation in those mice.

Pink-eyed dilution (*p/p*) mice produce greatly reduced (<1/10 that of black) levels of eumelanic pigment in follicular melanocytes in vivo (Table 1). However, in the present study, the plasma levels of 6H5MI2C and 5-*S*-CD in *p/p* mice at 7.5 days were ninefold and fourfold greater than in black mice. These results may appear surprising at first but are consistent with our finding that cultured *p/p* melanocytes fail to accumulate eumelanin and pheomelanin in the cells but release most of them (>90%) to the culture medium (Hirobe *et al.*, 2003). The dramatic increases in the plasma levels of 6H5MI2C and 5-*S*-CD after birth are also consistent with our previous results on the *p/p* melanocytes (Hirobe *et al.*, 2003).

The present study has demonstrated that the measurement of plasma levels of 6H5MI2C and 5-*S*-CD (in mice) provides useful information on the type of pigmentation. Of particular significance is the extremely high level of these melanin-related metabolites in pink-eyed dilution mice, suggesting the failure to accumulate melanin (and melanin-related metabolites) within melanocytes. The recent discovery of *SLC24A5*, a human orthologue of the zebrafish *golden* mutation, has stimulated renewed interests in the genetic basis of normal pigmentation variation seen within populations (Sturm, 2006). Several mutations in *SLC24A5*, *SLC45A2* (a human orthologue of mouse underwhite gene), and *OCA2* (a human orthologue of pink-eyed dilution gene) were found to be associated with light skin colours (Sturm, 2006). SLC24A5, SLC45A2 (also called MATP), and P protein are now thought to have similar roles in promoting melanin deposition through maturation of the melanosome. It would be thus interesting to compare the plasma levels of 6H5MI2C and 5-*S*-CD across various populations to see whether their release to the blood seen in mice is also a case in humans.

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