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G．知的財産権の出願・登録状況

１．特許取得

（神保主任研究者）

1）神保孝一

Title: PHENOLIC AMINE AS DEPIGMENTING AND ANTIMELANOMA AGNETS; K. Jimbow, MD, PhD, FRCPC, Stiefel Inc., & University of Alberta（２種類の特許）1998年：PATENT #: 3178834(in Japan), 5925332(in USA), 2015197(in Canada), 46522(in Philippine), 9604333-6(in Singapore), 204254(in South Korea), 82105703(in Taiwan), 651823(in Australia), 2000年：Israel (106347)

2)東レとの特許

Title: メラノーマ標的ナノ微粒子(NPrCAP/ML)によるメラノーマ温熱免疫療法の開発

（平成16年11月30日までの研究結果データに基づく研究内容）

Patent：特許第36178834号公報　（特許文献）

（佐藤・田村分担研究者）

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２．実用新案登録

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３．その他

特になし

Ⅱ．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

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Ⅲ. 研究成果の刊行物・別刷

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

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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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Program – November 1–4, 2007

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Select abstracts plenary presentation – 15 min + 5 min discussion
Minisymposia presentations – 10 min + 5 min discussion

SMR is pleased to announce the selection of 9 poster presenters for Travel Awards. The recipients of the Travel Award were selected based on applicants abstracts that were reviewed and ranked by the steering committee for the 4th International Melanoma Congress. These awards, which are provided by the Melanoma Research Foundation (MRF) will be given during the meeting to: Olivier Bailet, S. Byron, John D'Orazio, Thomas Guadagno, Tobias Hohenauer, Mei-Yu Hsu, James Lister, Feng Liu, Stephanie Arndt.

At the 4th International Melanoma Congress, Wiley-Blackwell offers an award for the best poster. A judging committee consisting of the Editor-in-Chief and members of the Melanoma Section of *Pigment Cell & Melanoma Research* (formerly known as *Pigment Cell Research*) evaluates each poster and selects a winner based on the quality of the work. The winner of the award receives a certificate, a cash award of $500, and a copy of Ortonne et al.: The Pigmentary System. The winner will be announced at the SMR dinner during the conference.

metastatic process is of paramount importance. Melanomas are notorious for their ability to metastasize at minimal depths of invasion which may be related to a unique interaction of melanoma cells with their associated vasculature. In order to study the molecular pathways involved in melanoma metastasis, we have taken a reductionist view of this process by restricting our studies to a two-cell system evaluating tumor interactions with endothelial cells. These studies have allowed us to make striking observations of tumor-endothelial cell crosstalk and allowed us to define the molecular readouts for this interaction for metastatic melanoma cells using gene expression profiling. Here we present data indicating the critical signature genes involved in melanoma-endothelial cell crosstalk and suggest that such communication networks will be critical mediators of the metastatic process. We further expect that such critical mediators of melanoma metastasis will be important therapeutic targets for early stage disease at risk for metastasis and will present early data on our most promising therapeutic targets for melanoma metastasis.

IS – 41 Exploitation of Melanogenesis for Tyrosinase-Targeted DDS and Chemo-Thermo- Immunotherapy with NPrCAP-magnetite for Melanoma Patients; Rationale and Preliminary Clinical Evaluation.

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Management of malignant melanoma with distant metastases is a difficult challenge. Here we report our approaches in drug synthesis, in vivo growth inhibition of B16melanoma and preliminary clinical evaluation of metastatic melanoma patients for developing melanogenesis-targeted, drug delivery system (DDS) and chemo-thermo-immuno (CTI) therapy. This is based upon our previous reports which showed that N-propionyl derivative of cysteaminylphenol (NPrCAP) is a good tyrosinase substrate and selectively incorporated into melanoma cells and inhibit in vivo and in vitro melanoma growth. This study will show that NPrCAP with magnetite (M), when given intralesionally and exposed to alternating magnetic field (AMF), can provide a novel tyrosinase-targeted DDS to melanoma cells, inhibit their growth and stimulate immune reaction through heat shock protein (HSP). The selective uptake of NPrCAP/M by melanoma cells was observed by both local intralesional and systemic *ip* administration. Every IInd administration of NPrCAP/M for three times with AMF caused a marked growth inhibition of melanoma cells (B16F1) and increased life span (%ILS) of host mice. Importantly mice that received melanoma inoculation *sc* initially on one site of flank and treated with NPrCAP/M plus AMF were then re-challenged by the second melanoma inoculation on the opposite flank showed the complete rejection of melanoma growth with the IInd re-challenge inoculation. The same melanoma growth inhibition with the IInd re-challenge inoculation was observed in control mice receiving NPrCAP/M without AMF exposure, though the growth inhibition effect was less than those seen with AMF exposure. Preliminary evaluation of our clinical phase I/II study following this experimental protocol indicated the marked and significant regression of cutaneous metastatic lesions in stage III-IV patients.

IS – 42 Phase II Trial of Neoadjuvant Temozolomide in Melanoma Patients

P.B. Chapman, G. Shah, M. Brady, D.G. Coit

The goal of this study is to identify tumor characteristics associated with response of melanoma to temozolomide (TMZ) chemotherapy. Advantages of the neoadjuvant approach include availability of fresh-frozen tumor pre- and post-treatment for study, determination of in vivo drug sensitivity which can guide future treatment plans, and treatment of potential micrometastatic disease before it is detectable. To be eligible for this study, patients (pts) with palpable and measurable stage III or IV melanoma must be candidates for complete surgical resection. Exclusion criteria include prior chemotherapy or pts with uveal or mucosal melanoma. Pretreatment evaluation includes CT scan (chest/abd/pelvis), PET scan, routine blood tests including $CD4^+$/$CD8^+$ T cell quantification. A pre-treatment tumor biopsy is obtained and flash-frozen. Pts receive TMZ at 75 mg/m^2/day x 6 weeks followed by 2 weeks off and are clinically evaluated every 4 weeks. CT scans are repeated prior to each cycle. Pts are treated until best response and then undergo surgical resection. For stable disease, no more than 2 cycles are given. If progression of disease is detected at any time, the pt stops TMZ and proceeds to surgery. At the time of surgery, tumor is collected and flash-frozen. To date, 13 evaluable pts have been accrued. There have been 2 complete responses, 1 partial response, 1 too early to evaluate (response rate 25%). Tumor biopsies are being analyzed by oligonucleotide array using Affymetrix U133 plus 2.0 array and for MGMT promoter methylation. The neoadjuvant approach offers a unique opportunity to study tumor tissue before and after treatment. Our goal is to identify correlations with clinical outcomes. Support: Schering-Plough Corp.

IS – 43 Transcription Regulation in Melanoma: The Mitf Code and Melanoma Metastasis

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Increasing evidence suggests that the rapid metastasis characteristic of melanoma may reflect microenvironment-driven activation of an intrinsic migratory capacity, rather than the acquisition of metastasis-associated genetic mutations. Indeed, during development melanoblasts migrate from the neural crest in solely in response to environmental cues. Understanding what controls melanoma/melanoblast proliferation and migration/metastasis is key to understanding why current treatments fail and the identification of novel therapeutic targets. We recently identified the Microphthalmia-associated transcription factor MITF as a regulator of melanoma proliferation and invasiveness, and provided evidence for a 'rheostat' model to resolve the apparent paradox in which MITF can act both to promote and inhibit proliferation. In this model, low levels of MITF yield a non-proliferating but invasive, stem cell-like population, intermediate MITF activity results in proliferation, while high levels gives a 'differentiated' phenotype. Importantly, our results imply that the microenvironment will drive a dynamic and reversible switch, mediated by MITF activity, which will determine the invasive or proliferative potential of individual cells within a tumour. The existence of a slow-proliferating subpopulation of melanoma 'stem-cell like' cells, may underlie the resistance of melanoma to therapeutic intervention. Our current understanding of how MITF activity and expression is regulated and how that will impact on melanoma behaviour will be discussed.

Rab7 Regulates Maturation of Melanosomal Matrix Protein gp100/Pmel17/Silv

Akinori Kawakami[1], Fumio Sakane[2], Shin-ichi Imai[2], Satoshi Yasuda[2], Masahiro Kai[2], Hideo Kanoh[2], Hai-Ying Jin[1], Kuninori Hirosaki[1], Toshiharu Yamashita[1], David E. Fisher[3] and Kowichi Jimbow[1,4]

Melanosome biogenesis consists of multistep processes that involve synthesis of melanosomal protein, which is followed by vesicle transport/fusion and post-translational modifications such as glycosylation, proteolysis, and oligomerization. Because of its complexity, the details of the molecular mechanism of melanosome biogenesis are not yet fully understood. Here, we report that, in MMAc melanoma cells, wild-type (WT) Rab7 and its dominant-active mutant (Rab7-Q67L), but not its dominant-negative mutant (Rab7-T22N), were colocalized in the perinuclear region with granules containing Stage I melanosomes, where the full-length, immature gp100/Pmel17/Silv was present. It was also found that overexpression of Rab7-Q67L and, to a lesser extent, Rab7-WT increased the amount of proteolytically processed, mature gp100. However, Rab7-T22N did not show such an effect. Moreover, siRNA-mediated Rab7 knockdown considerably inhibited gp100 maturation. These results collectively suggest that the GTP-bound form of Rab7 promotes melanogenesis through the regulation of gp100 maturation in melanoma cells.

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INTRODUCTION

The Rab family of small G proteins are known to be key regulators of intracellular vesicle traffic, including exocytosis and endocytosis. In mammalian cells, more than 60 Rab members have been identified, and each member localizes at the surface of a distinct intracellular compartment (Pfeffer, 1999; Somsel Rodman and Wandinger-Ness, 2000; Takai *et al.*, 2001; Zerial and McBride, 2001). The Rab GTPases serve as molecular switches that are activated on guanosine triphosphate (GTP) binding and inactivated following nucleotide hydrolysis, concomitantly cycling between the cytosol (GDP-bound, inactive form) and the membranes (GTP-bound, active form). To facilitate vesicle traffic, the GTPases regulate one (or several) specific step of vesicle traffic via recruitment of the tethering/docking and fusion factors and the actin- and microtubule-based motor proteins (Wu *et al.*, 2001; Strom *et al.*, 2002; Harrison *et al.*, 2003; Kuroda *et al.*, 2003; Chabrillat *et al.*, 2005).

Rab7 is present on late endosomes and lysosomes, and controls membrane trafficking between early and late endosomes (Press *et al.*, 1998), and from late endosomes to lysosomes (Feng *et al.*, 1995; Meresse *et al.*, 1995; Vitelli *et al.*, 1997). Rab7 has also been implicated in lysosome biogenesis by regulating the heterotypic fusion between late endosomes and lysosomes, and the homotypic fusion of lysosomes (Bucci *et al.*, 2000). Recently, some Rab7 target proteins such as Rab-interacting lysosomal protein (Cantalupo *et al.*, 2001; Harrison *et al.*, 2003), Rab-interacting RING finger protein (Mizuno *et al.*, 2003), hVPS34/p150 (Stein *et al.*, 2003), proteasome α-subunit XAPC7 (Dong *et al.*, 2004), and oxysterol-binding protein homologue ORP1L (Johansson *et al.*, 2005) have been identified and characterized. In addition, we have recently identified Rab7 as a melanosome-associated protein, being involved in the transport of tyrosinase-related protein 1 (TYRP-1) and tyrosinase from the *trans*-Golgi network (TGN) to melanosomes (Gomez *et al.*, 2001; Hirosaki *et al.*, 2002).

Melanosome biogenesis consists of four stages of maturation with distinct morphological and biochemical characteristics that reflect subcellular processes of the transport of structural and enzymatic proteins and their subsequent three-dimensional organization as well as melanin deposition (Jimbow *et al.*, 2000). gp100/Pmel17/Silv is one of the most enigmatic proteins in melanosome biogenesis. After synthesis, gp100 is transported from the TGN to Stage I melanosomes, which contain internal membranous vesicles that resemble late-endosomal multivesicular bodies, and is cleaved into several fragments, which form the fibrillar matrix of the organelle

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Abbreviations: PBS, phosphate-buffered saline; LAMP-2, lysosomal-associated membrane protein 2; siRNA, small interfering RNA; TGN, trans-Golgi network; TYRP-1, tyrosinase-related protein 1; WT, wild-type; YFP, yellow fluorescent protein

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