fetal bovine serum; RPMI, RPMI1640 medium; PBS, phosphate-buffered saline; HSP, heat shock protein; TYRP-1, tyrosinase-related protein-1.

1. Introduction

Although early lesions of primary melanoma are curable by excision, treatment of metastatic melanoma is significantly more difficult and the current systemic therapies have little effect on the overall survival rate or period [1,2]. Melanin pigment is synthesized in cytoplasmic organelles called melanosomes, specifically present in differentiated melanocytes. Melanin precursors possess strong cytotoxicy toward various kinds of cells, including melanoma cells, when tyrosinase, a key enzyme of melanin biogenesis, is ectopically expressed [3]. Thus, therapy targeting melanogenesis and enhancing cytotoxicity can be highly effective for growth suppression and possible induction of systemic immune responses against malignant melanoma without significant systemic side effects.

We have employed chemical agents biologically unique to melanoma cells such as the sulfur-amine analogs of tyrosine, N-acetyl-4-*S*-cysteaminylphenol (NAc-4-*S*-CAP) and N-propionyl-4-*S*-cysteaminylphenol (NPr-4-*S*-CAP). They have been shown to be good substrates for tyrosinase [4-6] and to be selectively incorporated into melanoma cells, causing cytotoxicity against them and melanocytes [7-11]. However, it remains unclear whether NPr-4-*S*-CAP can induce cell death associated with the induction of host immune responses resulting in the tumor suppression in vivo.

In this study, we examined the molecular mechanism of NPr-4-*S*-CAP-mediated cytotoxicity toward melanoma cells by focusing on intracellular reactive oxygen species (ROS) and tested whether NPr-4-*S*-CAP could suppress transplanted primary and secondary B16F1 melanomas. We analyzed the molecular basis of B16F1-specific host immunity through the application of CD4- and CD8-specific antibodies. The relations between apoptosis of melanoma cells and ROS production by NPr-4-*S*-CAP and clinical implications for melanoma therapy with this compound are discussed.

2. Materials and Methods

2.1 Cell lines and culture

Murine fibroblast NIH3T3, murine melanoma B16F1, human pigmented melanoma 70W and G361, human non-pigmented melanoma TXM18 and SK-mel-24 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS) [12]. Human pigmented melanoma cell line M-1 was established from a surgical specimen of a Japanese patient [13] and maintained in RPMI1640 medium (RPMI, Gibco BRL) supplemented with 5% FBS. Murine lymphoma cell line RMA was cultured in RPMI1640 medium supplemented with 5% FBS [12]. Cells were incubated at 37℃ in a 5% CO_2 atmosphere.

2.2 Chemicals

NPr-4-S-CAP (MW=225 D) was kindly provided by Alberta University's Department of Dermatology (Canada). The compound was synthesized as described by Tandon et al. [9]. NPr-2-*S*-CAP, an inactive form of cysteaminylphenol used as a negative control [5], was synthesized as follows: 195 mg of 2-*S*-cysteaminylphenol, which was prepared by the reaction of phenol with cystamine in 47% HBr [14], was reacted with 1.5 ml of pyridine and 1 ml of propionic anhydride for 30 min at room temperature.

After evaporation of the reaction mixture under reduced pressure in a vacuum pump, colorless crystals of NPr-2-*S*-CAP were obtained in a quantitative yield. For the study, NPr-4-*S*-CAP and NPr-2-*S*-CAP were dissolved in propylene glycol (Wako, Osaka, Japan) at a concentration of 244 mM and sterilized by filtration.

2.3 Animal models for tumor formation

Female C57BL/6J mice (4 weeks old, approximately 10.0 g) were obtained from Hokudo (Sapporo, Japan). For the experiment, 3.0×10^5 B16F1 melanoma cells in 0.1 ml of phosphate-buffered saline (PBS) were injected subcutaneously into the right flank in C57BL/6J mice on day 0. On day 8, 24 mice were randomly divided into four treatment groups. From the 8th day, tumor-bearing mice were injected with 0.1 ml of NPr-4-*S*-CAP (24.4 mmol, 5.5 mg) in propylene glycol directly into the tumor with a 26-gauge microsyringe. Group I mice received s.c. administration of NPr-4-*S*-CAP every other day for a total of three days (days 8, 10, and 12) and administration of 0.1 ml of propylene glycol on days 14 and 16. Group II mice received NPr-4-*S*-CAP every other day for a total of five days (days 8, 10, 12, 14, and 16), and in group III NPr-4-*S*-CAP was administered every day for five days (from day 8 to day 12). Control mice were injected with 0.1 ml of propylene glycol every day for five days (Protocol 1). To compare the anti-melanoma effect of NPr-4-*S*-CAP with that of NPr-2-*S*-CAP, mice were divided into another three groups on day 8, and injected with 0.1 ml of NPr-4-*S*-CAP, NPr-2-*S*-CAP or propylene glycol every day for five days (Protocol 2). RMA lymphoma cells (5.0×10^5) were injected subcutaneously into the right flank on day 0 as a control for B16F1 melanoma and, from day 8, mice received NPr-4-*S*-CAP or propylene glycol every day for five days (Protocol 3). Tumor diameters were measured every other day and tumor volumes were calculated using the following formula: long axis $\times$ (short axis)^2 $\times$ 0.5. The right tumors of treatment and control groups in Protocol 1 were resected surgically on day 22. On day 36, mice were rechallenged with 1.5×10^5 B16F1 cells that were injected into the left flank and tumor diameters were measured every other day from the 37th through 120th days. As a control for B16F1 rechallenge, 2.5×10^5 RMA lymphoma cells in 0.1 ml of PBS were injected subcutaneously into the left flank in group III on day 36.

Animal experiments were carried out according to the principles described in the "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology" of Japan.

2.4 Histopathological study of tumor sections

The tumors were removed and fixed in 10% formalin in PBS. Paraffin-embedded sections were then prepared and processed for Hematoxylin-eosin (HE)-staining. For immunohistochemical analysis, the frozen tissues were stained with anti-mouse CD4 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-mouse CD8 mAb (Acris Antibodies, ACR, Herford, Germany).

2.5 *In vivo* T-cell depletion assay

Mice that received NPr-4-S-CAP every day for five days Protocol 1 were injected with 0.2 mg of an anti-CD4 antibody (anti-mouse CD4, eBioscience, San Diego, CA, USA), anti-CD8 antibody (anti-mouse CD8, eBioscience) or rat IgG (Rat IgG1, aBd Serotec, Kidlington, UK) i.p. on days 29 and 43, i.e., one week before and after the rechallenge test on day 36. The growing tumor diameters were measured

and tumor volumes were calculated.

2.6 *In vitro* cytotoxicity assay

After mice were treated with NPr-4-*S*-CAP every day for five times as described above, the spleen cells were harvested from a mouse which was completely cured by day 36 by NPr-4-*S*-CAP. 5 × 106 spleen cells were then re-stimulated with irradiated B16F1 cells in 2 ml of RPMI1640 supplemented with 50 × M × -mercaptoethanol (Invitrogen, Carlsbad, CA, USA) and 5% FBS for five days. For the control, spleen cells were prepared from a naïve mouse and a mouse transplanted with RMA lymphoma cells and treated similarly with NPr-4-*S*-CAP. Cytotoxic activity of spleen effector cells against target B16F1 or RMA cells was determined by standard ^{51}Cr release assay.

2.7 Tetramer Assay

Spleen cells were removed on day 40 after primary melanoma resection. The cells were stimulated with TRP-2 peptide (2 mg/ml) for five days. Collected splenocytes were incubated at 37℃ for 30 min in the staining buffer (PBS with 0.1% BSA and 0.1 % sodium azide) containing 10 ■ l of APC-labeled TRP-2-specific tetramer (Medical & Biological Laboratories CO.,Ltd., MBL, Nagoya, Japan). Cells were washed once, then incubated at 4℃ in the staining buffer containing 3 ■ g of anti-CD8a mAb conjugated to FITC (Becton, Dickinson and Company (BD), NJ, USA). Samples were analyzed by FACS vantage using CellQuest software (BD). Lymphocytes were gated on $CD8^+$ cells. More than 105 events were acquired for each sample.

2.8 Cell proliferation assay

For assessment of the growth-inhibitory activities of NPr-4-*S*-CAP and NPr-2-*S*-CAP, NIH3T3, B16F1, 70W and M1 cells were dispensed at a density of 1.0 × 10^5 in 6-cm dishes and cultured for 24 hrs. Selected concentrations of NPr-4-*S*-CAP (0.5, 1.0, 2.0, and 3.0 mM), NPr-2-*S*-CAP (0.5, 1.0, 2.0, and 3.0 mM) or propylene glycol (120 μ l) were added. After cells were cultured for 1 hr, they were washed and refed with fresh DMEM and cultured for 24 hrs. The numbers of living cells that were not stained by trypan blue were counted and the average of 6 dishes was determined.

2.9 Flow cytometric analysis

B16F1, NIH3T3, RMA and TXM18 cells were cultured in the NPr-4-S-CAP-containing medium (1 mM) or propylene glycol for 1 hr, they were washed in PBS and cultured for 24 hrs. For the positive control of apoptosis, TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L (Wako, 2.0 μg/ml) was added and the cells were cultured for 24 hr. Next, adherent and floating cells were collected together, washed in PBS, dehydrated in 70% cold ethanol and stored on ice for 2 hrs. They were then rehydrated in cold PBS and incubated in the presence of RNaseA (100 μg/ml) (Invitrogen) at 37℃ for 30 min. After incubation, the cells were rinsed twice in cold PBS and suspended in 2.0 ml of PBS containing 10 μg/ml propidium iodide (Wako) at 4℃ for 2 hrs. Sub G1, G1, S, and G2/M populations were quanified with a FACS Calibur flow cytometer (BD) using the Cell QUEST program.

2.10 Caspase enzyme assay

For this assay, 1.5 × 10^4 B16F1, NIH3T3, RMA and TXM18 cells were seeded in 96-well plates. After 24 hrs, the medium was replaced by one containing NPr-4-S-CAP or propylene glycol at the indicated concentrations followed by incubation for 1 hr at 37℃ and washing with PBS. For positive control, cells were cultured in the presence of TRAIL/Apo2L and cultured for 24 hrs. After medium containing

Caspase-Glo3/7 Assay kit solution (100 μl, Promega, Madison, WI) was added and the cells were cultured for two hrs, activity of caspase 3/7 was measured using a Wallac 1420 ARVO series multilabel reader (Perkin Elmer, MA, USA).

2.11 TUNEL assay

In order to assess the apoptosis in NPr-4-*S*-CAP mediated melanoma tumors, TUNEL assay was performed with an apoptosis detection kit (Takara Bio, Shiga, Japan). Tumors in group III and control groups processed according to Protocol 1 and 3 were harvested on day 22 as described above and fixed in formalin (4% w/v) overnight. Samples were then dehydrated and embedded with paraffin. The staining was operated according to the manufacturer's instructions.

2.12 Fluorescence microscopy

Using a confocal fluorescence microscope (Radiance 2000MP, BIO-RAD, CA, USA), we studied the morphologic features of cell death induced by NPr-4-*S*-CAP. For this, 1.5 × 10^4 B16F1 and NIH3T3 cells were separately seeded on round glass coverslips that were coated with Atelo Cell IPC-30 (Koken Co.,Ltd., Tokyo, Japan) and put into 12-well plates. They were cultured for 24 hrs with 5% FBS at 37℃ in a 5% CO_2 atmosphere. Cells were stained with 5 μM 5-(and-6)- chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen) for 30 min. They were then cultured in the medium containing NPr-4-*S*-CAP (1.0 mM) or propylene glycol (40μl) for 1 hr. After being cultured for 24 hrs, they were stained with a Rab pAb to Active Caspase 3 (Abcam, Cambridge, MA, USA), stained with Hoechst33342 (10 μl per dish, Wako, Japan) and analyzed under a confocal microscope. The difference between apoptosis and necrosis was determined according to a previous report [15]; cells with nuclei showing aggregation and deformation were considered to be apoptotic and those with round nuclei to be necrotic.

2.13 Detection and analysis of intracellular ROS

After non-pigmented melanoma TXM18 and SK-mel-24 cell lines and pigmented melanoma B16F1, 70W, M-1 and G361 cells were cultured in medium containing 5 μM CM-H2DCFDA (DCF, invitrogen) for 30 min at 37℃ , they were washed with PBS, refed with medium containing NPr-2-*S*-CAP (1, 3, 6 mM) or NPr-4-S-CAP (0.5, 1, 2, 3, or 6 mM). After they were cultured for 1 hrs, they were washed in PBS and cultured for 24 hrs. For the positive control with exposure to ROS, the cells were cultured with H_2O_2 (0.5, 1, 2, 3 mM) for 24 hr. Next, adherent and floating cells were collected together and processed for flow cytometry to quantify the M2 fraction using the Cell QUEST program (BD).

2.14 Statistical analyses

The data were analyzed by one- or two-way analysis of variance (ANOVA) and then differences in experimental results for tumor growth were assessed by Sheffe's test to compare all the experimental groups, or Dunnett's test for the experimental groups vs. the control group. For multiple comparisons the data were assessed by the log-rank test with Bonferroni correction. Differences in survival rates were analyzed by the Kaplan-Meier method. The level of significance was $P<0.05$ (two-tailed). All statistical analyses were performed using StatView J-5.0 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 NPr-4-S-CAP induces apoptotic cell death of B16F1 cells

B16F1, 70W and M-1 melanoma and NIH3T3 fibroblast cells were separately cultured in media containing different concentrations of NPr-4-*S*-CAP (0-3.0 mM) and NPr-2-*S*-CAP (0-3.0 mM). After a 24hr-incubation period, the anti-proliferative effect was assessed by cell-counting assay. Figure 1 shows the effects of different concentrations of NPr-4-*S*-CAP and NPr-2-S-CAP on melanoma and NIH3T3 cells. The results indicated that NPr-4-*S*-CAP had a dose-dependent antiproliferative effect on B16F1, 70W and M-1 cells ($p<0.05$, 51.6% of the relative cell number at the concentration of 3.0 mM in B16F1 cells) but not on NIH3T3 cells (83.7% at 3.0 mM). Conversely, NPr-2-*S*-CAP, an inactive form of CAP, did not have antiproliferative effects on B16F1 or NIH3T3.

To examine the mechanism of the cell death induced by NPr-4-*S*-CAP, these cells were subjected to flow cytometric analysis, caspase 3 assay and TUNEL staining. The sub-G1 fraction was increased in the NPr-4-*S*-CAP-treated B16F1 cells, comparable to TRAIL-exposed B16F1, but not in the NPr-4-*S*-CAP-treated non-melanoma cells (NIH3T3, RMA) or non-pigmented melanoma cells (TXM18) (Figure 2A). The luminescent assay detected caspase 3/7 activity in the NPr-4-*S*-CAP-treated B16F1 cells remarkably increased (35.8-fold) compared to that in the non-treated cells (Figure 2B). NIH3T3, RMA and TXM18 cells treated with TRAIL showed 10.6, 7.1 and 5.8-fold increases of caspase 3/7 activation compared to the control, respectively, whereas those with NPr-4-*S*-CAP showed increases of 4.1, 1.4 and 1.8-fold, respectively (Figure 2B). As shown in Figure 2C, the number of TUNEL-positive cells was significantly increased only in the B16F1 tumor treated with NPr-4-*S*-CAP. This increase was not observed in the B16F1 tumor without NPr-4-*S*-CAP or in RMA tumors with or without NPr-4-*S*-CAP. These findings suggested that NPr-4-*S*-CAP induces apoptotic cell death selectively in pigmented melanoma cells.

3.2 NPr-4-*S*-CAP produces ROS

Melanoma cells produce ROS in the process of melanogenesis, possibly resulting in their degradation with disruption of cell membranes [16-18], but the molecular mechanism of NPr-4-*S*-CAP-mediated cell death has not yet been elucidated. To analyze the relations between NPr-4-*S*-CAP-mediated apoptosis and ROS production in B16F1 cells, we examined ROS generation and morphological changes using confocal fluorescent microscopy and flow cytometer. When B16F1 cells were cultured in the medium containing NPr-4-*S*-CAP, about half of their nuclei were aggregated and deformed, which was associated with an increase of ROS and caspase 3 in their cytoplasm (Figure 3). NIH3T3 cells, however, did not show any of these changes (Figure 3). When pigmented (B16F1, 70W, M-1 and G361) and non-pigmented (TXM18 and SK-mel-24) melanoma cells were treated with NPr-4-*S*-CAP, ROS production was selectively observed in the pigmented melanoma cell lines (Figure 4). These findings suggested that NPr-4-*S*-CAP selectively produced ROS in pigmented melanoma cells such as B16F1 and that ROS played an important role in the NPr-4-*S*-CAP-associated apoptosis.

3.3 NPr-4-S-CAP suppresses growth of primary and secondary B16F1 tumors in mice

To examine whether NPr-4-*S*-CAP had an anti-melanoma effect, syngeneic mice bearing B16F1 tumors were given NPr-4-*S*-CAP by intratumoral injections and the volumes of tumors were measured as described in Methods. First, we compared the direct effects of NPr-4-*S*-CAP and NPr-2-*S*-CAP on

the transplanted B16F1 melanomas. Growth of tumors injected with NPr-4-*S*-CAP was remarkably suppressed (P=.0247 by a Sheffe test), but those treated with NPr-2-*S*-CAP, like control mice, were not affected (Figure 5). The mice were divided into four different groups, a non-treated control group, and treated groups I, II and III. Tumors in group II (injected every other day for a total of five times) and group III (injected for five consecutive days), especially, showed significant reductions in their volumes compared to those of control mice by day 20 (P=.0163 and .0100, respectively, vs. control group, Figure 6A). On the other hand, NPr-4-*S*-CAP did not affect the tumor volume of transplanted RMA T-cell lymphoma (Figure 6B). These results suggested that NPr-4-*S*-CAP had a selective chemotherapeutic effect on B16F1 melanoma cells.

We then examined whether secondary B16F1 tumors transplanted on the opposite flank were suppressed after the treatment of primary tumors with NPr-4-*S*-CAP. In all the treated groups (I, II and III), tumor growth after rechallenge with secondary B16F1 melanomas was suppressed (Figure 6C). The tumor volumes of groups II and III were significantly reduced compared to those of the control mice (P=.0321 and .0160, respectively, Figure 6C). However, secondary RMA T-cell lymphoma tumors were not suppressed when they were transplanted after the primary B16F1 tumors were removed (Figure 6D). The survival periods and survival rates of mice among the four groups were observed for as long as 120 days. Significant prolongations of survival were observed in groups I, II and III compared to the control group (P=.0177, .0024 and .0435, respectively, Figure 7A).

In the histopathological examination against primary and secondly transplanted B16F1 or RMA tumors, a dense inflammatory infiltrate including neutrophils, macrophages, plasma cells and lymphocytes was observed around NPr-4-*S*-CAP treated B16F1 tumors which were harvested on day 22 (Figure 8D, E). Furthermore, we observed that numerous $CD8^+$ T cells were infiltrated within these tumors (Figure 8G). By contrast, inflammatory cells were not detectable in the primary B16F1 tumors without treatment or RMA tumors with or without treatment (Figure 8A, B, C, data not shown for RMA tumors). In the secondly B16F1 tumors, the inflammatory cells were infiltrated around the residual tumor but the density of $CD4^+$ and $CD8^+$ T cells was not prominent as the treated primary tumors (data not shown).

3.4 $CD8^+$ T cells mediate suppression of rechallenge with B16F1 melanoma

Six weeks after the NPr-4-*S*-CAP injections, vitiligo (depigmented cutaneous lesions) or a change in hair color to white appeared in two of the six mice in group III in areas of the body distant from the injection points (Figure 7B). This suggested that the vitiligo was not generated by a direct effect of NPr-4-*S*-CAP but by the induction of systemic tumor-specific T-cell immunity against melanocytes. To analyze the mechanism of the anti-melanocyte immunity induced by NPr-4-*S*-CAP, in vivo T cell depletion assay was performed. When B16F1 melanoma-bearing mice were injected with an anti-CD8 antibody i.p., tumor growth was not suppressed. However, the growth of tumors remained significantly suppressed (i.e., it was not further affected) after injection of an anti-CD4 antibody or Rat IgG i.p. (P=.0167 vs. Rat IgG group, Figure 9A). This suggested that $CD8^+$ T cells participated in the NPr-4-*S*-CAP -mediated anti-B16F1 immunity.

To analyze whether tumor-specific immunity had been induced, CTL induction was examined by Cr-release assay and tetramer assay. Spleen cells were obtained from the cured mouse and mixed

with B16F1 cells to detect their specific lyses. As shown in Figure 9B, spleen cells showed cytotoxic activity to B16F1 cells but not to the non-melanoma RMA cells. However, spleen cells from the naïve or RMA-challenged mice did not show cytotoxic activity to either B16F1 or RMA cells. For the tetramer assay, splenocytes were prepared from a naïve mouse and from mice with B16F1 or RMA tumors after treatment with NPr-4-*S*-CAP, and stimulated *in vitro* with TRP-2 peptide for five days. Stimulated splenocytes were then stained with TRP-2-specific tetramer, and proportions of peptide-specific $CD8^+$ T cells were determined. As shown in Figure 9C, 9.08 % of the $CD8^+$ T cells from the B16F1-challenged and NPr-4-*S*-CAP-treated mouse were specific for the TRP-2 peptide, compared with 2.66 % or 2.96 % from the naïve or RMA-challenged mice, respectively. The results from the two independent CTL assays were highly consistent, showing that the NPr-4-*S*-CAP treatment to B16F1 melanoma produced a functional CTL response.

4. Discussion

Although selective cytotoxicities and anti-melanoma effects of cysteaminyl phenol derivatives have been reported, the mechanisms of cell death induced by the compounds have not been elucidated. Here, we demonstrated that NPr-4-*S*-CAP induced apoptosis selectively in pigmented melanoma cells in association with ROS production and caspase 3 activation. In addition, this is the first report demonstrating that NPr-4-*S*-CAP can induce host T-cell immune responses associated with rejection of in vivo rechallenge with melanoma.

Tyrosinase inhibitors with simple skeletal structures similar to phenol such as hydroquinone, kojic acid, arbutin and deoxyarbutin have been used as skin whitening agents via topical application [19]. Administration of a melanin precursor such as 4-*S*-cysteaminylphenol to black mice resulted in depigmentation of hair follicles, possibly by the selective disintegration of melanocytes in the hair bulb [10,14,20-22]. NPr-4-*S*-CAP exerts strong cytotoxicity toward melanoma cells, in which melanin synthesis is highly elevated [8,9,11]. When phenolic amine compounds are oxidized by tyrosinase, melanin intermediates inhibit the activity of SH enzymes such as thymidylate synthase, alcohol dehydrogenase and DNA polymerase by covalent binding through their cysteine residues, resulting in melanoma-specific cytotoxicity [23-25].

In the present study, NPr-4-*S*-CAP suppressed growth of cultured melanoma cells and induced apoptotic cell death accompanied by ROS generation. Since pigmented melanoma cells (B16F1, 70W, G361 and M-1), but not non-pigmented ones (TXM18 and SK-mel-24), produced significant amounts of ROS (Figure 4) in the presence of NPr-4-*S*-CAP, it was suggested that melanin biosynthesis was essential for NPr-4-*S*-CAP to produce ROS. It has been reported that ROS-mediated apoptosis is initiated via two pathways, the death receptor (Fas ligand-Fas receptor) and the mitochondria-mediated pathway in which the Trx/ASK1 complex acts as a redox switch to release cytochrome C [26,27]. Intracellular ROS mediate apoptotic cell death, which elicits a strong host inflammation reaction and subsequent induction of host immune responses [28]. Transcription factor NF-E2-related factor 2 (Nrf2) is activated by ROS to induce cellular genes such as glutathione-S-transferase, heme oxygenase and glucose-6-phosphodehydrogenase to protect cells from oxidative stress [29,30]. It is possible that Nrf2-1 and/or its related genes could not be induced to a level sufficient for the protection of melanoma cells

from oxidative stress.

In the mouse model of thermotherapy for B16 melanoma, it has been suggested that HSPs, especially HSP70 and HSP72, are involved in tumor-specific CTL responses [31,32]. We suggested in a previous report that the CTL response against B16-OVA melanoma induced by hyperthermia using NPrCAP/M was associated with the release of the HSP72-peptide complex from degraded tumor cells [33]. In the present study, we therefore examined whether HSPs were induced in and released from melanoma cells after injection of NPr-4-*S*-CAP; however, Western blot analysis did not detect an increase of HSP72 or HSP90 in the NPr-4-*S*-CAP-treated B16F1 cells (data not shown). Thus, although intracellular HSPs may play a role in the induction of NPr-4-*S*-CAP-mediated antitumor immunity, the induction of HSPs is not a prerequisite for the NPr-4-*S*-CAP-mediated tumor-specific immune response.

Results of the in vivo T cell depletion assay using anti-CD8 antibodies, Cr-releasing assay and tetramer assay using B16F1 and spleen cells from NPr-4-*S*-CAP-treated and cured mice suggested that suppression of the primary tumor by NPr-4-*S*-CAP was able to induce $CD8^+$ T cell immunity against component of B16F1 melanoma. Melanoma tumor antigens recognized by T lymphocytes are derived from melanocyte differentiation antigens (tyrosinase, TYRP-1 and gp100), tumor testis antigens (NY-ESO-1, MAGE1 and MEGE3) and mutated cellular gene products (β-catenin and p53). They have been used for immunotherapy against malignant melanoma through vaccination with defined antigens [34]. Although it is not clear which B16F1 tumor antigens were presented on the dendritic cells for induction of $CD8^+$ T cells, a melanocyte differentiation antigen(s) was responsible for both the depigmentation and inhibition of the rechallenge tumor growth. Recently, Westerhof et al. proposed the haptenation theory in which increased intracellular H_2O_2 could trigger the increased turnover of elevated levels of surrogate substrates of tyrosinase, resulting in melanocyte-specific T-cell responses [35,36]. According to this hypothesis, tyrosinase would be recognized as a melanoma-specific tumor antigen in relation to the systemic immune responses. This line of study may contribute to the clinical application of NPr-4-*S*-CAP for therapy targeting malignant melanoma.

Acknowledgments

This work was supported by Health and Labor Sciences Research Grants-in-Aid (H17-nano-004 and H21-nano-006) for Research on Advanced Medical Technology from the Ministry of Health, Labor and Welfare of Japan.

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Figure Legends

Figure 1. NPr-4-*S*-CAP, but not NPr-2-*S*-CAP, suppresses growth of melanoma cells. The number of cells cultured in the medium containing NPr-4-*S*-CAP or NPr-2-*S*-CAP was counted as described in Materials and Methods. All data are presented as mean ± standard deviation. Growth of B16F1, 70W and M-1 melanoma, but not NIH3T3 fibroblast cells, was suppressed by NPr-4-S-CAP (P<.05, Dunnett's test).

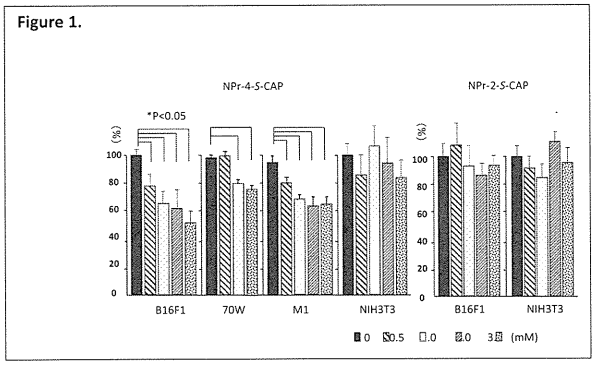


Figure 2. NPr-4-*S*-CAP mediated apoptotic cell death of B16F1 melanoma cells. (A) Flow cytometric analysis of cellular DNA detected an increased sub-G1 fraction in NPr-4-*S*-CAP-treated B16F1 (a), but not NIH3T3 (b), RMA (c) or TXM18(d). (B) Assay of caspase3/7 in cells treated with NPr-4-*S*-CAP or TRAIL. Cells were cultured in the presence of NPr-4-*S*-CAP, TRAIL, or propylene glycol in 96-well plates and then processed for measurement of caspases 3 and 7 using a Caspase-Glo3/7 Assay kit. (C) Apoptosis of B16F1 tumors induced by NPr-4-*S*-CAP was assessed by the TUNEL method. Samples were obtained from (a) B16F1 tumors injected with propylene glycol, (b) B16F1 tumors treated with NPr-4-*S*-CAP, (c) RMA tumors injected with propylene glycol and (d) RMA injecteds with NPr-4-*S*-CAP. TUNEL-positive nuclei appear dark brown. (X200)

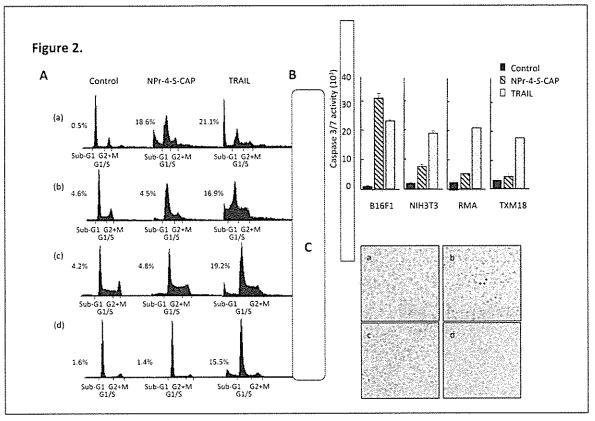


Figure 3. Confocal microscopic observation of cells treated with NPr-4-*S*-CAP. B16F1 and NIH3T3 cells were stained with CM-H_2DCFDA for ROS production, a Rab pAb for activated caspase 3, and Hoechst 33342 for living nuclei.

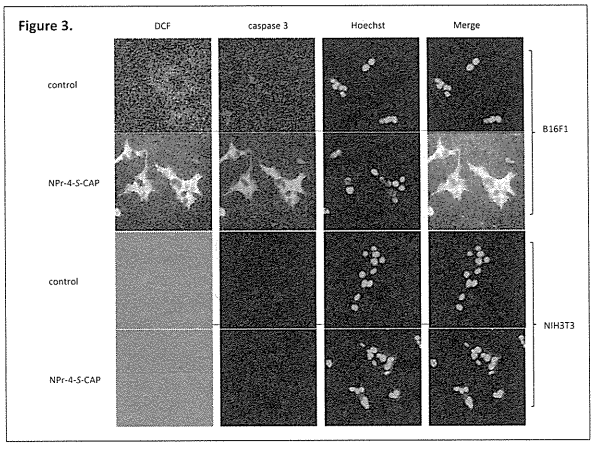


Figure 4. Pigmented melanoma but not non-pigmented melanoma cells produce ROS in the presence of NPr-4-*S*-CAP. Cells were cultured in the presence of MDCF for 30 min, washed twice

with PBS, refed with medium containing NPr-2-*S*-CAP or NPr-4-*S*-CAP and cultured for a further 1 hr. They were then harvested and processed for FACScan analysis.

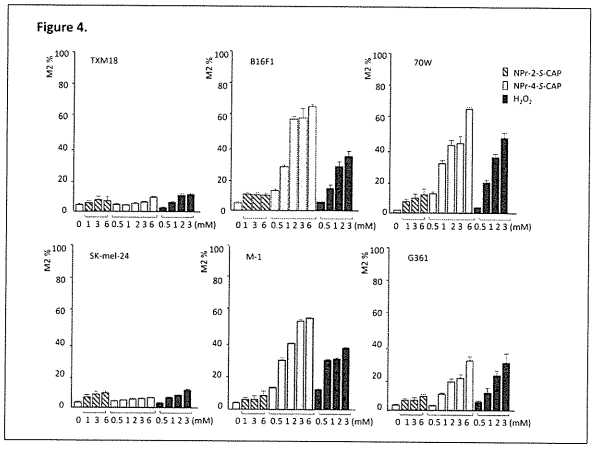


Figure 5. NPr-4-*S*-CAP, but not NPr-2-*S*-CAP, suppresses growth of transplanted B16F1 tumors. The syngeneic mice bearing B16F1 tumors were given 24.4 μmol NPr-4-*S*-CAP or NPr-2-*S*-CAP in five consecutive intratumoral injections and the volumes of tumors were measured.

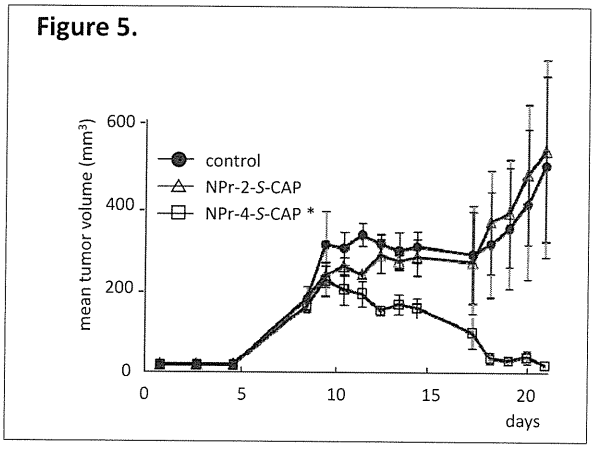


Figure 6. NPr-4-*S*-CAP suppresses growth of primary and secondary rechallenge B16F1 melanomas. (A) Mice were transplanted with B16F1 cells and treated with NPr-4-*S*-CAP. Tumor volumes of treated groups I, II and III were suppressed. In particular, tumors in groups II and III showed significant reductions in their volumes ($P<.05$). (B) Transplanted RMA mouse T lymphomas were injected with NPr-4-S-CAP and tumor volumes were measured. (C) Tumor volumes of secondary B16F1 melanomas in mice belonging to groups I, II and III. Growth of rechallenge melanomas on the opposite flanks was markedly suppressed in the mice of groups II and III. (D) Growth of secondarily transplanted RMA lymphomas was not inhibited in mice after the primary B16F1 tumors were removed.

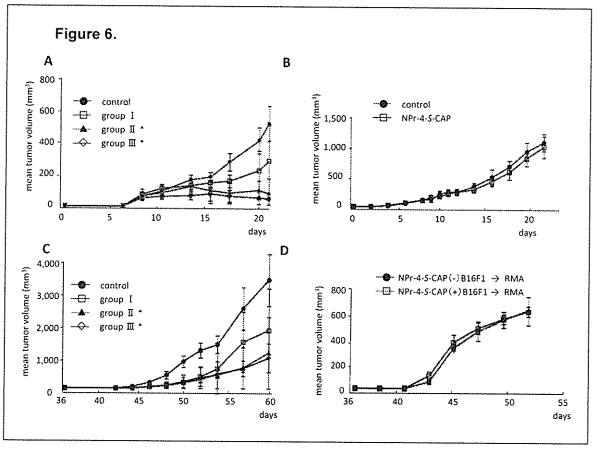
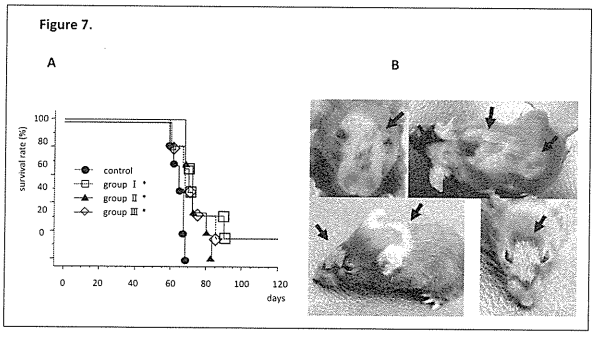


Figure 7. (A) The survival periods and survival rates of tumor-bearing mice. Kaplan-Meier survival curves over a period of 120 days after the tumor transplantation showed that significant prolongations of survival were observed in groups I, II and III compared to the control mice



(P=.0177, P=.0024 and P=.0435, respectively, vs. control). **(B) Appearance of vitiligo on the treated mice.** Six weeks after the NPr-4-S-CAP injection, vitiligo or white hair appeared at body sites distant from the injection point.

Figure 8. Dense inflammatory cells and CD8$^+$ T cells were infiltrated in NPr-4-*S*-CAP treated B16F1 tumors. The B16F1 tumors were harvested on day 22 and histopathological features were analyzed by HE-staining (A, D, E) or an anti-mouse CD4 mAb (B, F) or CD8 mAb (C, G).

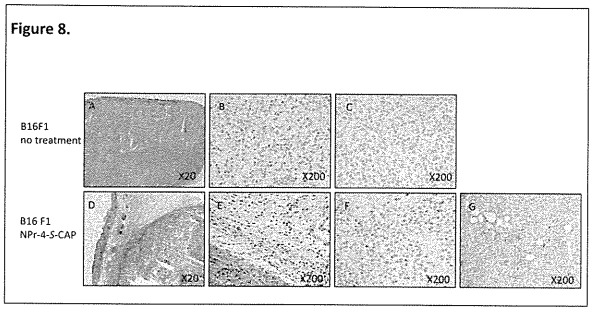


Figure 9. CD8+ T cell immunity was induced in the treated mice. (A) Suppression of secondary tumors is inhibited by an anti-CD8 antibody. After the first challenge, the mice from group III were injected with 0.2 mg of anti-CD4, anti-CD8 or rat IgG i.p. on days 29 and 43, one week before and after the rechallenge by B16F1 transplantation, respectively. **(B) Spleen cells of tumor-bearing mice contain CTL activity against B16F1 cells.** After the first challenge, the spleen cells were harvested from the mice and cytotoxic activity of effector cells against target cells (B16F1 and RMA) was determined by standard ^{51}Cr assay. T: tumor B16F1 cells, E: effector spleen cells. **(C) Detection of murine CTL using tetramer assays.** Splenocytes from naïve mice (control), RMA- or B16F1-challenged mice treated with NPr-4-*S*-CAP after in vitro stimulation with TRP-2 peptide were stained with APC-labeled TRP-2-specific tetramer together with FITC-labeled anti-CD8 mAb. The numbers shown represent the percentage of tetramer+ cells within CD8$^+$ T lymphocytes.