N-Propionyl-Cysteaminylphenol-Magnetite Conjugate (NPrCAP/M) Is a Nanoparticle for the Targeted Growth Suppression of Melanoma Cells

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A magnetite nanoparticle, NPrCAP/M, was produced for intracellular hyperthermia treatment of melanoma by conjugating *N*-propionyl-cysteaminylphenol (NPrCAP) with magnetite and used for the study of selective targeting and degradation of melanoma cells. NPrCAP/M, like NPrCAP, was integrated as a substrate in the oxidative reaction by mushroom tyrosinase. Melanoma, but not non-melanoma, cells incorporated larger amounts of iron than magnetite from NPrCAP/M. When mice bearing a B16F1 melanoma and a lymphoma on opposite flanks were given NPrCAP/M, iron was observed only in B16F1 melanoma cells and iron particles (NPrCAP/M) were identified within late-stage melanosomes by electron microscopy. When cells were treated with NPrCAP/M or magnetite and heated to 43°C by an external alternating magnetic field (AMF), melanoma cells were degraded 1.7- to 5.4-fold more significantly by NPrCAP/M than by magnetite. Growth of transplanted B16 melanoma was suppressed effectively by NPrCAP/M-mediated hyperthermia, suggesting a clinical application of NPrCAP/M to lesional therapy for melanoma. Finally, melanoma cells treated with NPrCAP/M plus AMF showed little sub-G1 fraction and no caspase 3 activation, suggesting that the NPrCAP/M-mediated hyperthermia induced non-apoptotic cell death. These results suggest that NPrCAP/M may be useful in targeted therapy for melanoma by inducing non-apoptotic cell death after appropriate heating by the AMF.

Journal of Investigative Dermatology (2009) **129,** 2233–2241; doi:10.1038/jid.2009.39; published online 19 March 2009

INTRODUCTION

Although early lesions of primary melanoma are curable by excision, successful treatment of metastatic melanoma has been elusive thus far. The current systemic therapies have little effect on the overall survival period or rate of advanced melanoma (Balch *et al.*, 2001). Because melanogenesis is inherently toxic and uniquely expressed in melanocytic cells, tyrosine analogs can be good candidates for melanoma-specific targeting and therapy (Jimbow *et al.*, 1993). To develop melanocytotoxic compounds for rational chemotherapy for melanoma, *N*-acetyl-cysteaminylphenol and *N*-propionyl-cysteaminylphenol (NPrCAP) were synthesized. These compounds showed selective cytotoxicity against melanoma cells *in vivo* and *in vitro* (Jimbow *et al.*, 1984; Miura *et al.*, 1990; Alena *et al.*, 1994; Tandon *et al.*, 1998). They have both cytostatic and cytocidal effects on melanoma cells (Thomas *et al.*, 1999), and induce apoptosis in follicular melanocytes of mice (Minamitsuji *et al.*, 1999). Thus, these synthetic compounds would provide the basis for the development of novel anti-melanoma agents.

Iron oxide and magnetite nanoparticles are becoming versatile tools for medical imaging of lymph nodes and are excellent candidates for hyperthermia induced by an external alternating magnetic field (AMF) due to the loss of hysteresis (Leary *et al.*, 2006; van Vlerken and Amiji, 2006). Local hyperthermia is induced in tumors by injecting magnetite nanoparticles into the core of the solid tumor and AMF irradiation results in shrinkage of animal tumors (Luderer *et al.*, 1983; Minamimura *et al.*, 2000). Magnetite cationic liposomes (MCL) have been generated for the selective accumulation of magnetite nanoparticles in tumor tissues,

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Abbreviations: 4-S-CAP, 4-S-cysteaminylphenol; AMF, alternating magnetic field; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MCL, magnetite cationic liposome; NPrCAP, N-propionyl-(4-S-) cysteaminylphenol; PBS, phosphate-buffered saline

Received 7 February 2008; revised 26 December 2008; accepted 14 January 2009; published online 19 March 2009

and MCL-mediated hyperthermia has inhibited growth or induced complete regression of various tumors in the transplanted animals (Yanase *et al.*, 1998; Ito *et al.*, 2003; Kawai *et al.*, 2005). If the magnetite nanoparticle technology is taken a step farther to achieve a selective delivery system to tumors, guided hyperthermia could be achieved for treatment of metastatic tumors.

Recently, we synthesized an MCL in which NPrCAP was encapsulated within the liposomes, resulting in both intracellular hyperthermia and cytotoxicity when injected into animal melanoma (Ito *et al.*, 2007). Here, we introduce another magnetite nanoparticle, NPrCAP/M, to which NPrCAP was superficially bound to enhance its targeting activity to melanoma cells. The possible mechanisms of NPrCAP/M-mediated hyperthermia against melanoma are discussed.

RESULTS

Incorporation of *N*-(1-mercaptopropionyl)-4-*S*-cysteaminylphenol (NPrCAP-SH) with magnetite

The degree of incorporation of NPrCAP-SH with magnetite was determined by HCl hydrolysis of NPrCAP/M followed by HPLC analysis of the 4-*S*-cysteaminylphenol (4-*S*-CAP) produced. We measured 4-*S*-CAP as an index of the degree of NPrCAP-SH incorporation as they share the same structural units. The results indicated that the degree of incorporation of NPrCAP-SH with magnetite was 405 nmol mg^{-1} magnetite. When B16F1 cells were cultured in NPrCAP/M-containing medium, collected, and exposed to the AMF generator, the temperature rose sharply from 30 to 50°C within 10 minutes and decreased immediately after the machine was switched off (Figure 1).

NPrCAP/M as substrate for tyrosinase

We examined whether NPrCAP/M could act as a substrate for tyrosinase. 4-*S*-CAP itself was found to be a good substrate for mushroom tyrosinase; tyrosinase oxidation of 4-*S*-CAP (100 μM) in the presence of cysteine yielded 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol through *ortho*-quinone within 10 minutes. HPLC showed that the reaction was almost completed within 10 minutes with half of the 4-*S*-CAP remaining after 4.2 minutes. At the same time, the expected catechol derivative was produced at 85 μM (85% yield) at 10 minutes. As NPrCAP/M has the same structural units as 4-*S*-CAP, it was expected to be a substrate for tyrosinase. If this were the case, 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol would be obtained by HCl hydrolysis of the cysteinylcatechol derivative of NPrCAP/M produced after tyrosinase oxidation of NPrCAP/M in the presence of cysteine. NPrCAP/M fell to half of the initial concentration after 69 minutes, and the concentration of 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol produced after 3 hours was 80 μM (80% yield) (Figure 2). Thus, the ratio of 4-*S*-CAP to NPrCAP/M in the reaction velocity on tyrosinase oxidation was 16. These results indicate that NPrCAP/M served as a substrate for mushroom tyrosinase.

Measurement of the magnetite incorporated into cells treated with NPrCAP/M

To examine whether NPrCAP/M could be incorporated into melanoma cells more preferentially than magnetite alone, we compared amounts of iron molecules in cells after culture in the NPrCAP/M- or magnetite-containing medium. To prevent non-specific adsorption of the particles to the cells, culture flasks were filled with NPrCAP/M-containing medium and rotated. After cells were collected and lysed, the amount of iron was measured. As shown in Figure 3, MM418, 70W, B16F1, SK-mel-23, TXM18, AK-1, and 96E melanoma cells incorporated large amounts of iron derived from NPrCAP/M compared with that from magnetite alone. Primary human melanocytes and non-pigmented SK-mel-24 and SK-mel-118 cells captured a relatively large amount of NPrCAP/M; however, the amount was not significantly different from that from magnetite treatment or almost the same as for magnetite (Figure 3).

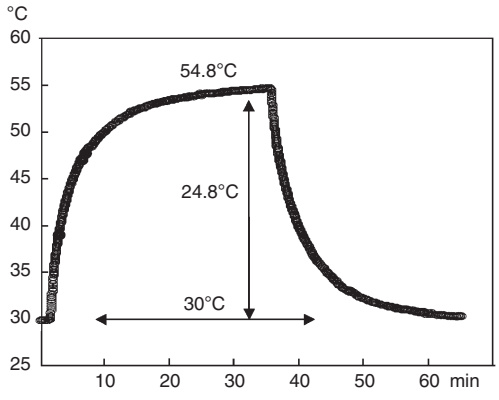

Figure 1. Heat generation in cells treated with NPrCAP/M and irradiated by AMF. 2×10^6 B16F1 cells were cultured in NPrCAP/M (5.0 mg magnetite equivalent)-containing medium for 30 minutes, collected, and exposed to the center of the coil of the AMF generator. The temperature at the center of the cell pellets was measured using an optical fiber probe. A rapid increase and decrease in temperature were observed in the cell pellet during AMF irradiation.

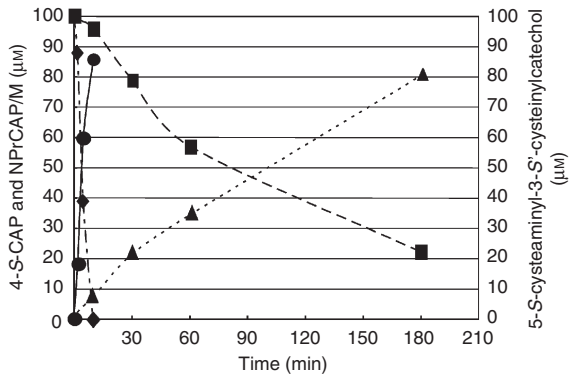

Figure 2. NPrCAP/M is incorporated into the tyrosinase oxidative reaction *in vitro*. The concentrations of the substrate remaining as 4-*S*-CAP and the 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol produced were measured by HPLC analysis after hydrolysis with HCl. ◆: 4-*S*-CAP, ■: NPrCAP/M, ●: 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol from 4-*S*-CAP, ▲: 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol from NPrCAP/M.

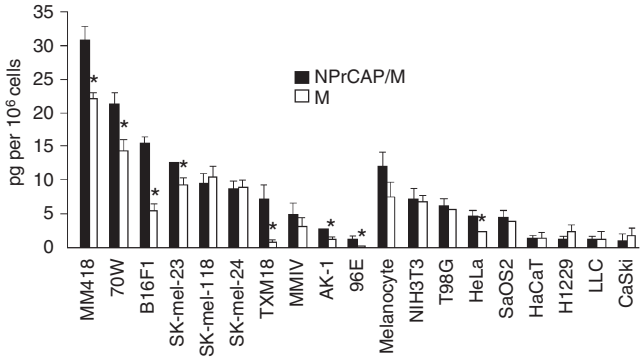

Figure 3. Uptake of magnetite nanoparticles into melanoma and non-melanoma cells. 75 cm^2 flasks containing growing cells were filled with NPrCAP/M- or magnetite-containing medium and fixed on a slanted disc, which was rotated slowly for 30 minutes. Incorporated iron was measured by the potassium thiocyanate method. Melanoma cell line names (MM418 to 96E) are written in gothic. Data and bars are mean ± SD of three independent experiments (*$P<0.05$).

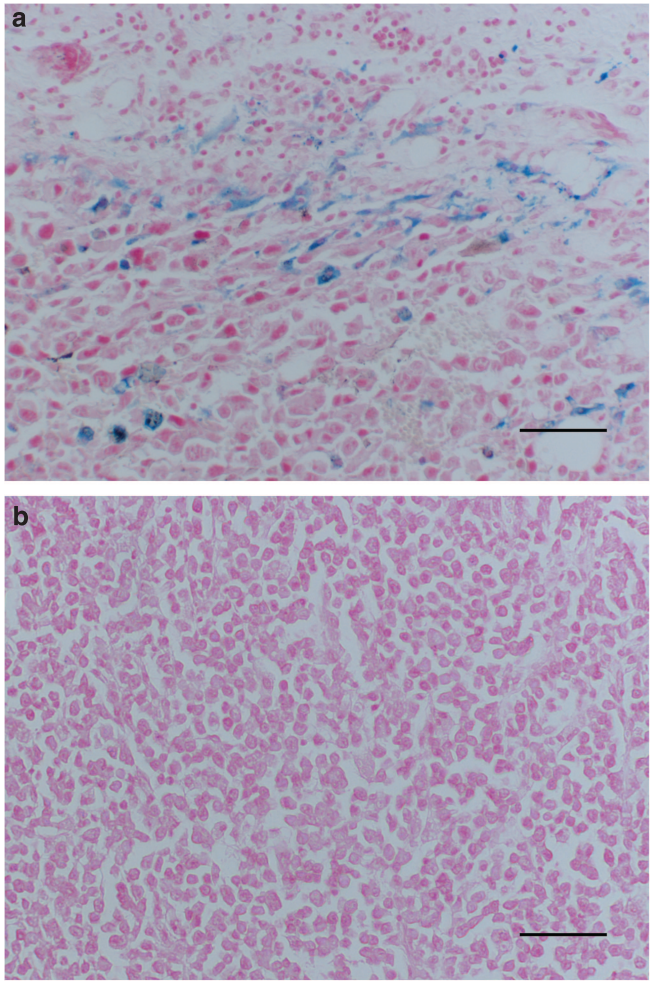

Figure 4. Intraperitoneal NPrCAP/M nanoparticles were delivered to the subcutaneously transplanted melanoma tumors. Mice bearing B16F1 and lymphoma tumors received i.p. administration of NPrCAP/M and were maintained for 14 days. Tumors were then removed and processed for hematoxylin–eosin and Berlin blue staining. Iron-containing blue-stained tumor cells were detected in the B16F1 tissues (**a**), but not in the EG7 (**b**) or RMA lymphoma tissues. Data are for five or four independent mice, each. Bars represent 50 μm (**a**, **b**).

NPrCAP/M is delivered to transplanted B16F1 melanomas

We then tested whether NPrCAP/M could be delivered to B16F1 melanoma tumors transplanted into syngeneic C57BL/6 mice. In five sets of experiments, each of which consisted of three to five mice, we transplanted a B16F1 melanoma onto the left flank and an EG7 or RMA lymphoma onto the right flank, and we injected NPrCAP/M or magnetite into the intraperitoneal cavity. After being allowed to grow for 2 weeks, tumors were excised and examined for the presence of iron (NPrCAP/M) by Berlin blue staining. Blue-stained cells were detected in 11 of the 14 melanomas, but in none of the 14 lymphomas (Figure 4a and b, Table 1). Meanwhile, in the B16F1- and EG7-bearing mice given magnetite, blue-stained tumor cells were not detected in either the melanoma or lymphoma tissues.

B16F1 melanomas were removed and examined for the subcellular localization of iron particles by electron microscopy. B16F1 cells in the NPrCAP/M-injected mice contained iron particles within dense ellipsoidal organelles, corresponding to late-stage melanosomes (data not shown). This suggested that NPrCAP/M was finally delivered to the melanogenesis system of the melanocytic cells.

Cytotoxic effects of magnetically mediated hyperthermia on melanoma cells

Because melanoma cells preferentially take up NPrCAP/M, it was expected that NPrCAP/M-treated melanoma cells would be selectively degraded by the AMF irradiation. MM418, SK-mel-23, B16F1, and TXM18 melanoma and H1229, HaCaT, HeLa, and SaOS2 non-melanoma cells were cultured in the NPrCAP/M- or magnetite-containing medium, collected, and irradiated by AMF at 43°C for 30 minutes. Figure 5 shows the results for NPrCAP/M- or magnetite-treated cells with or without hyperthermia induced by AMF. All the melanoma cells tested were degraded more significantly by NPrCAP/M with AMF than by magnetite with AMF, with differences ranging from 1.7-fold in SK-mel-23 to 5.4-fold in B16F1 cells (Figure 5a), whereas non-melanoma cells were degraded almost equally by NPrCAP/M and magnetite (Figure 5b). These results suggested that NPrCAP/M could induce the death of melanoma cells more selectively and significantly than that of non-melanoma cells at the relatively low temperature of 43°C.

Hyperthermia mediated by NPrCAP/M effectively suppresses growth of mouse melanoma

To evaluate whether NPrCAP/M-mediated hyperthermia could suppress melanoma in the mouse model, we treated the subcutaneously transplanted B16F1 melanoma and measured the volumes of tumors (Figure 6). As shown in Figure 6a, B16F1 melanoma in mice treated by magnetite injection followed by AMF irradiation and NPrCAP/M injection followed by AMF irradiation resulted in statistically significant suppression of tumor growth compared with the untreated melanoma (Figure 6a and b). NPrCAP/M-mediated hyperthermia seemed to suppress growth of the melanoma

more than hyperthermia mediated by magnetite alone; however, differences between the two groups were not statistically significant.

Table 1. Presence of iron-containing tumor cells in mice injected with NPrCAP/M or magnetite

	Number of mice bearing a Berlin-blue positive tumor/number of total mice tested		
	B16F1	EG7	RMA
Exp I NPrCAP/M	5/5	0/5	NT
Exp II NPrCAP/M	3/4	NT	0/4
Exp III NPrCAP/M	3/5	0/5	NT
Exp IV magnetite	0/3	0/3	NT
Exp V magnetite	0/3	NT	0/3

Mice bearing B16F1 melanoma and EG7 or RMA lymphoma on the left and right flanks, respectively, were intraperitoneally given NPrCAP/M or magnetite. Tumors were excised and the presence of iron was examined by Berlin-blue staining.

Non-apoptotic cell death is induced by NPrCAP-mediated intracellular hyperthermia

Cellular DNA was prepared after cells had been cultured in the NPrCAP/M-containing medium followed by AMF at 43°C and subjected to analysis by flow cytometry. B16F1 and SK-mel-23 cells infected with a recombinant adenovirus expressing Ad-p63′ showed evident sub-G1 fractions, whereas cells subjected to NPrCAP/M-mediated hyperthermia contained little sub-G1 DNA (Figure 7). Levels of caspases 3, 8, and 9 in B16F1 and SK-mel-23 cells after NPrCAP/M-mediated hyperthermia were as low as those without NPrCAP/M or after NPrCAP/M treatment without hyperthermia (Figure 8). These results suggested that NPrCAP/M-mediated hyperthermia induced non-apoptotic cell death or necrosis.

DISCUSSION

The temperature at the center of the pellet of NPrCAP/M-treated B16F1 cells rose to over 50°C within 10 minutes; thus, the NPrCAP/M was a good heat generator, comparable to MCL or 4-S-CAP-loaded magnetite (Shinkai *et al.*, 1996;

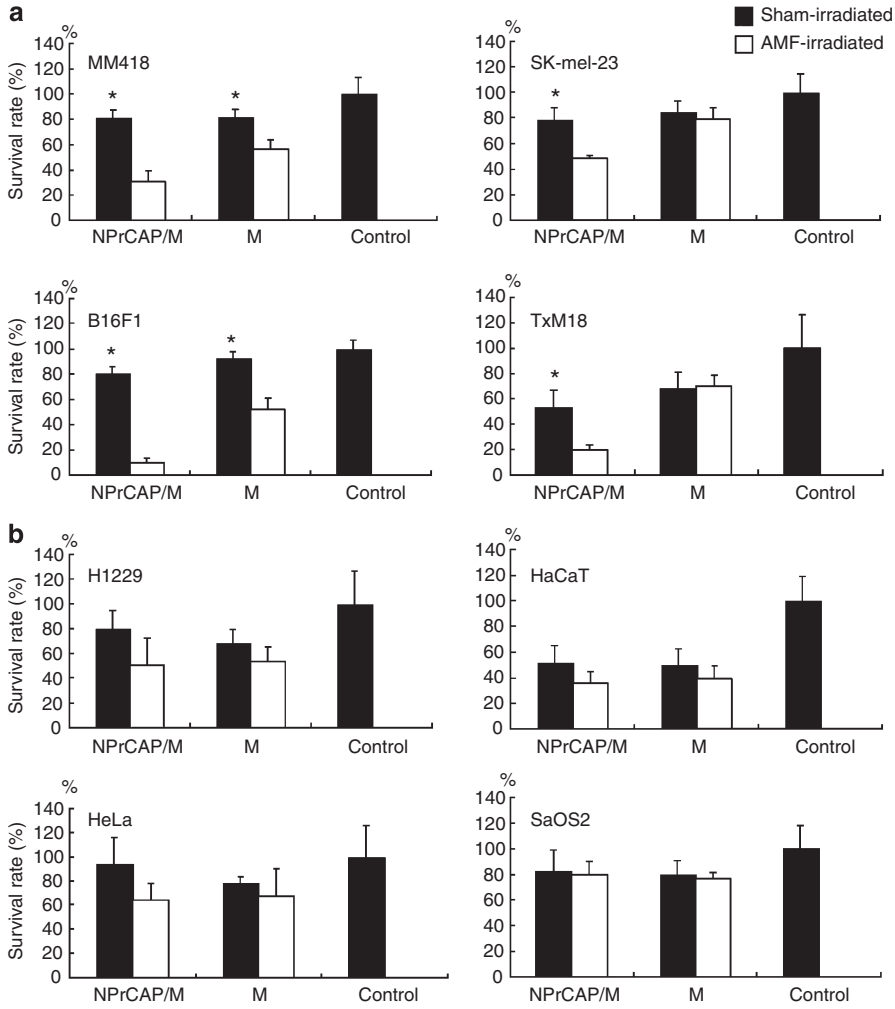

Figure 5. NPrCAP/M plus AMF treatment degraded melanoma cells more significantly than NPrCAP/M without the AMF. After cells were cultured in the NPrCAP/M-containing medium and collected, cell pellets were exposed to sham (■) or AMF (□) irradiation. Treated cells were collected and the number of viable cells not stained by trypan blue was counted. Data and bars are mean ± SD of three independent experiments (*$P<0.05$). (**a** and **b**) show results for melanoma and non-melanoma cell lines, respectively.

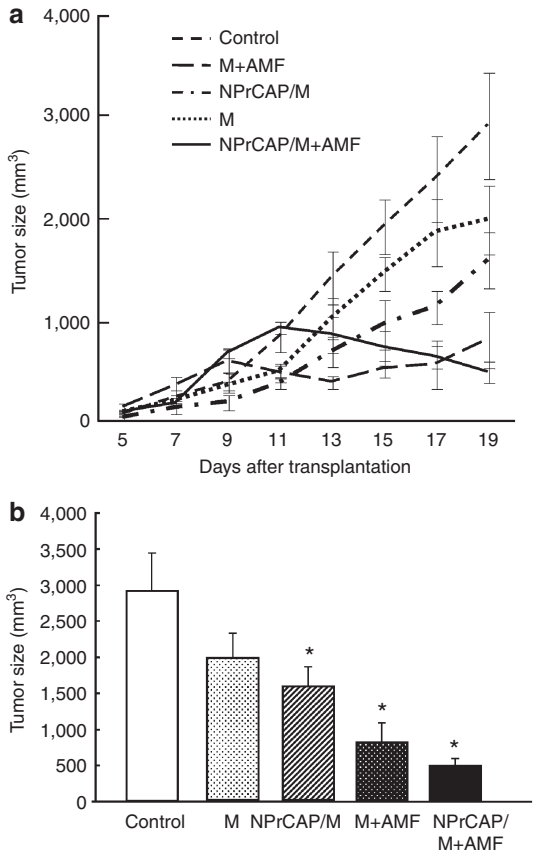

Figure 6. Tumor volumes of the B16F1-bearing mice treated by magnetite- and NPrCAP/M-mediated hyperthermia. (**a**) Comparison of groups in the first 19 days after tumor transplantation. Magnetite or NPrCAP/M (4 mg of magnetite or its equivalent) was injected directly into subcutaneous B16F1 tumors, which were then irradiated with an AMF at 46°C for 30 minutes. Each point represents the mean ± SD of five mice. All data are presented as mean ± SD. (**b**) Comparison of tumor volumes in each group on the 19th day. *$P<0.005$, tumors treated by NPrCAP/M with the AMF, magnetite (M) with the AMF, and NPrCAP/M without the AMF were significantly different from those of control mice.

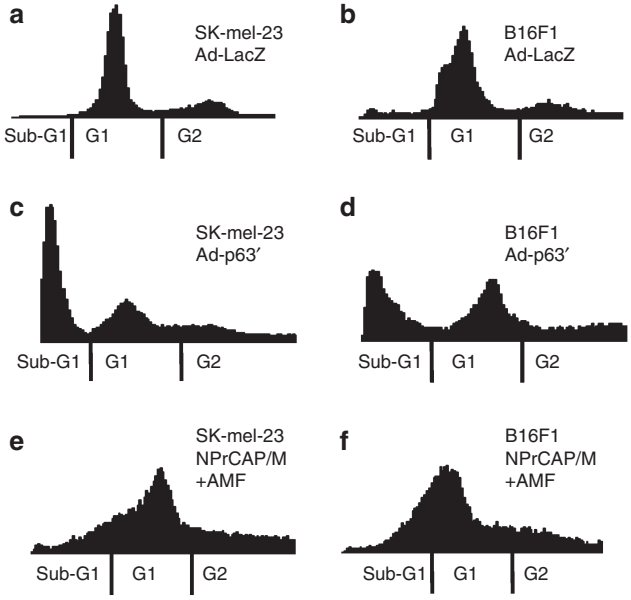

Figure 7. The sub-G1 fraction was not observed in NPrCAP/M-treated and AMF exposed cells. After cells were treated with NPrCAP/M followed by AMF irradiation and culture for 24 or 48 hours, adherent and floating cells were collected and sub-G1, G1, S, and G2/M populations were quantified with a FACScan Cell Sorter. (**a**): SK-mel-23 with Ad-LacZ, (**b**): B16F1 with LacZ, (**c**): SK-mel-23 with Ad-p63′, (**d**): B16F1 with Ad-p63′, (**e**): SK-mel-23 with NPrCAP/M + AMF, (**f**): B16F1 with NPrCAP/M + AMF.

Yanase *et al.*, 1997; Ito *et al.*, 2007). To examine NPrCAP/M as a tyrosinase substrate, we could not use the spectrophotometric assay owing to the brown suspension of the substrate. Thus, we used a method based on the fact that *ortho*-quinone obtained from tyrosinase oxidation of the substrate can be trapped with cysteine, and we monitored the cysteine adduct with HPLC. Tandon *et al.* (1998) have reported that NPrCAP is a very good substrate for tyrosinase and the enzyme's kinetic parameters (K_m and V_{max}) were found to be similar to those of the homolog *N*-acetyl-4-*S*-CAP. They also reported that the K_m values for 4-*S*-CAP and NPrCAP were 117 and 340 μM, whereas the V_{max} values were 39.0 and 5.4 μmol min^{-1} mg^{-1} of protein, respectively. In this study, the ratio of 4-*S*-CAP to NPrCAP/M in the reaction velocity on tyrosine oxidation was 16. The reaction velocity was not as good as for 4-*S*-CAP (Figure 2). However, it should be efficient enough, if we consider the bulky structure of NPrCAP/M.

Pigmented melanoma cells, such as B16F1, MM418, 70W, and SK-mel-23, captured larger amounts of iron from NPrCAP/M than from magnetite (Figure 3). These pigmented melanoma cells also captured magnetite particles without NPrCAP, although the amount of iron from the magnetite was lower than that from NPrCAP/M. It is unclear why non-pigmented AK-1 and 96E and non-melanoma HeLa cells incorporated NPrCAP/M more efficiently than magnetite. It is possible that an unidentified receptor for cysteaminyl-phenols might be present on the surface or in the cytoplasm of the cells. When mice bearing B16F1 melanoma were intraperitoneally injected with NPrCAP/M, a total of 11 of 14 melanoma tissues on 14 mice contained B16F1 cells showing Berlin blue iron staining. As NPrCAP/M was injected into the peritoneal cavity in the mice, the nanoparticles were delivered to the B16 melanoma in the subcutis through the bloodstream. In the B16F1 tumors in the mice injected with magnetite, blue-stained cells were detected in the encapsulating fibroblast-like cells, but not in the tumor cells, suggesting that NPrCAP/M, but not magnetite, was preferentially delivered to the B16F1 cells. However, a large part of NPrCAP/M given i.p. was captured in reticuloendothelial cell systems such as the liver and spleen in the mice (data not shown). Clinical trials using the present magnetite-NPrCAP nanoparticles might be limited to lesional therapy against melanoma. We have proceeded to a phase I/II study of the effect of NPrCAP/M-mediated hyperthermia not only on treated tumors but also on non-treated metastatic tumors.

Hyperthermia reduces cell viability and proliferation in a time- and temperature-dependent manner in melanoma

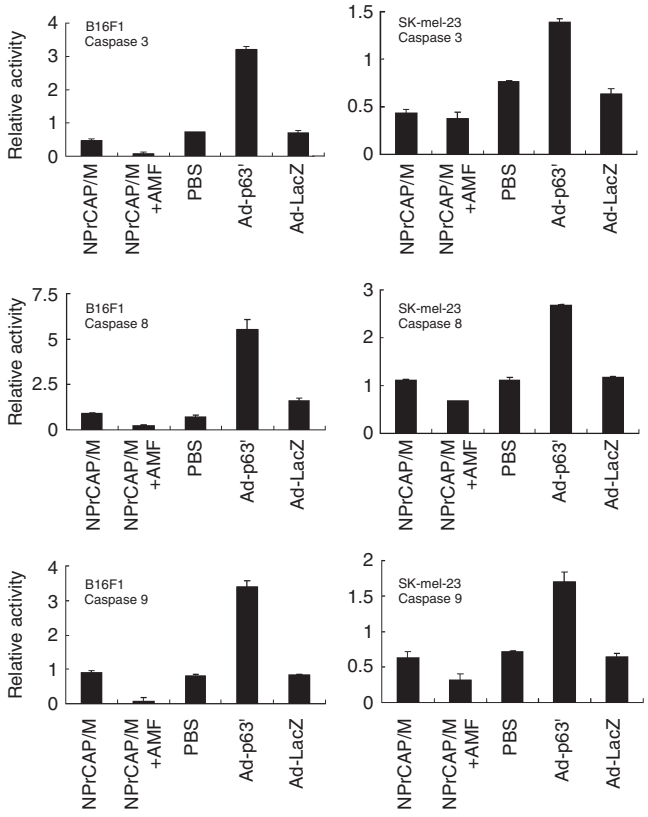

Figure 8. Caspases 3, 8, and 9 were not activated in cells that received NPrCAP/M-mediated hyperthermia. B16F1 mouse melanoma cells and SK-mel-23 human melanoma cells were treated with NPrCAP/M with or without hyperthermia. Cells were infected with 20 pfu per cell of Ad-p63′ or Ad-LacZ for 24 hours for the positive control of apoptotic cell death. Cells were collected and processed for assay of caspase activities. Error bars represent the mean ± SD from two separate experiments.

cells (Shellman *et al.*, 2007). Intracellular hyperthermia of NPrCAP/M-treated cells resulted in a significant degradation of melanoma cells (Figure 5a). No difference was found in the cell numbers of non-melanoma cell lines between NPrCAP/M- and magnetite-treated dishes (Figure 5b). These results were comparable to those of an iron-incorporation assay of cells cultured in NPrCAP/M- and magnetite-containing media (Figure 3), suggesting that the targeting ability of NPrCAP/M to melanocytic cells determined the degree of cell degradation. In a previous study, significantly higher therapeutic effects were observed in mice treated with 4-*S*-CAP/MCL + AML (43°C) than in those treated with 4-*S*-CAP/MCL or hyperthermia alone (Ito *et al.*, 2007). In our animal-model experiments, growth of transplanted B16F1 tumors was suppressed more effectively than in the untreated control. Because untreated control mice died on the 19th and 21st days after inoculation, survival curves over a period of 60 days could not be compared. However, two and one mice that received NPrCAP/M- and magnetite-mediated hyperthermia survived to the 60th day after treatment, respectively, whereas mice belonging to the other groups did not. Therefore, NPrCAP/M- and magnetite-mediated hyperthermia was suggested to be more effective for tumor suppression than treatment without heat. Interestingly, NPrCAP/M injection without heat suppressed B16 melanoma more than in the control mice (Figure 6b), suggesting that NPrCAP possessed intrinsic cytotoxicity against melanoma cells. Another experimental system that can evaluate targeting, permeating, and suppressive abilities of NPrCAP/M needs to be designed.

Necrotic, but not apoptotic, cell death is believed to induce inflammation and immunity in the host (Shellman *et al.*, 2007). When HL-60 cells were cultured at 43°C for 1 hour, cell degradation was observed in association with cellular DNA fragmentation and activation of caspases 3 and 8 (Han *et al.*, 2007). It has also been reported that hyperthermia at 41–44°C promotes TRAIL-induced apoptosis by facilitating caspase activity, whereas hyperthermia at 45–46°C inhibits this type of apoptosis (Yoo and Lee, 2007). In contrast to *in vivo* observation of apoptotic cell death of follicular melanocytes in NPrCAP-injected mice (Minamitsuji *et al.*, 1999), we found no evidence of apoptosis in cells treated with NPrCAP/M-mediated hyperthermia at 43°C. None of the caspase 8, caspase 9, or caspase 3 required for the execution of the final phase of apoptosis was activated in cells treated with NPrCAP/M and irradiated with the AMF. Although it is unclear why NPrCAP/M-mediated hyperthermia at a relatively low temperature (43°C) induced non-apoptotic cell death, this thermotherapy might elicit systemic T-cell immunity in advanced melanoma. Evidence along this line has been obtained by our group (Sato *et al.*, manuscript submitted for publication).

HSPs are molecular chaperones in the cytoplasm upregulated by various stress stimuli that damage proteins and promote accumulation of misfolded proteins (Brostrom and Brostrom, 1998). HSP70 was efficiently produced and excreted from B16F1 cells treated with NPrCAP/M and heated at 43°C compared with those heated at 46°C (data not shown). When transplanted B16F1 melanoma in mice was treated with NPrCAP/M-mediated hyperthermia at 43°C for 30 minutes, rechallenged melanoma was clearly suppressed (Takada *et al.*, manuscript submitted for publication). In this animal model system, the first tumors treated at 43°C contained larger amounts of HSP70 than those treated at 46°C. Judging from these results, HSP70 was produced most abundantly by the NPrCAP/M-mediated hyperthermia at 43°C, and the combination therapy with NPrCAP/M plus hyperthermia at this temperature resulted in the most significant therapeutic effect on advanced melanoma.

MATERIALS AND METHODS

Cell lines and cell culture

Human cancer cell lines (T98G, HeLa, SaOS2, HaCaT, H1229, and CaSki), human melanoma cell lines (SK-mel-23, SK-mel-24, SK-mel-118, MM418, 70W, TXM18, MMIV, AK-1, and 96E), murine melanoma cell line B16F1, and fibroblast cell line NIH3T3 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS) and antibiotics. Murine lymphoma cell lines EG7, LLC and RMA were cultured in RPMI-1640 supplemented with 5% FBS. Primary human epidermal melanocytes (BioWhittaker Inc., Walkerville, MD, USA) were grown in the basal medium supplemented

with basic FGF, hydrocortisone, TPA, insulin, and bovine pituitary extract according to the manufacturer's instructions.

Establishment of tumors in mice

Female C57BL/6 mice (age 4 weeks, weighing approximately 10.0 g) were purchased from Hokudo Co. Ltd. (Sapporo, Japan). Cell suspensions containing approximately 1×10^6 B16F1 melanoma, EG7, or RMA lymphoma cells in 0.1 ml of phosphate-buffered saline (PBS) were injected subcutaneously into C57BL/6 mice after anesthesia by diethyl ether. B16F1 melanoma and EG7 or RMA lymphoma cells were injected subcutaneously into the left and right flanks of C57BL/6 mice, respectively. Mice with tumors received intraperitoneal administration of 123.8 mg of NPrCAP/M on the third day after tumor injection. At the 15th day after transplantation, tumors were removed and processed for Fe staining. The Committees for Animal Research of Sapporo Medical University approved the experimental protocols of this research project.

Animal treatment model

Mouse B16F1 melanoma cells (5.0×10^5) in 0.1 ml of PBS were inoculated s.c. into the right flanks of 4-week-old female C57BL/6 mice. On day 5 after inoculation, mice were randomly divided into control and treatment groups. Each group was composed of five mice. With a 26-G microsyringe, the B16F1 melanoma-bearing mice were injected with 0.1 ml of NPrCAP/M or magnetite (40.0 mg ml^{-1} solution) directly into the tumor site in a single-dose administration on days 5, 7, and 9. Hyperthermia was carried out on days 6, 8, and 10; mice were exposed to the AMF inside the coil and heated at 46°C for 30 minutes. Temperatures on the tumor surface were measured using an optical fiber probe (FX-9020; Anritsu Meter, Tokyo, Japan). Tumor size was measured every other day for 60 days by the formula: long axis × (short axis)^2 × 0.5. Data were analyzed by one- or two-way analysis of variance, and then differences in experimental results for tumor growth were assessed by Sheffe's test to compare all the experimental groups, or by Dunnett's test, which compared the experimental *versus* the control groups.

Iron oxide and chemicals

Magnetite nanoparticles (Fe_3O_4; average particle size, 10 nm) were kindly provided by Toda Kogyo (Hiroshima, Japan). 4-*S*-CAP was prepared as described by Padgette *et al.* *N*-succinimidyl-3-[2-pyridyldithio] propionate and mushroom tyrosinase (6050 U mg^{-1}) were obtained from Molecular Biosciences, Inc. (Boulder, CO) and Sigma Chemical Co. (St Louis, MO), respectively. 3-Aminopropyl-triethoxysilane and *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester were products of Tokyo Chemical Industry (Tokyo, Japan) and Pierce (Rockford, IL), respectively. 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 *N*-succinimidyl-3-[2-pyridyldithio] propionate (13.2 mmol) in 5 ml of pyridine was stirred for 2 hours at room temperature. After evaporation, the residue was purified by silica gel column chromatography (ethyl acetate:n-hexane; 2:1 v/v as eluant) to give a disulfide (3.70 g; 94%). Then 4.29 g of dithiothreitol (27.5 mmol) was added to a stirred solution of the disulfide (3.70 g; 10.3 mmol) in 5 ml of methanol at room temperature. After 2 hours the mixture was evaporated and the oily residue was purified by 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 recrystallization (ethyl acetate ether). The elemental analysis of NPrCAP-SH was as follows: Anal. Calcd for $C_{11}H_{15}N_1O_2S_2$: C, 51.36; H, 5.84; N, 5.45; S, 24.90; Found: C, 51.41; H, 5.78: N, 5.50; S, 24.83. The resulting material was subjected to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis using an electrospray ionization/ion trap mass spectrometer (LCQ Deca XP, Thermoelectron, Tokyo, Japan). The analysis was carried out directly by MS/MS at a positive charge; $[M+H]^+$: *m/z* 258, 164, 153, 132, 125. ^{1}H NMR was measured at 400 MHz (CD_3COCD_3): 2.45 p.p.m. (2H, t, $J = 6.8$ Hz), 2.71 p.p.m. (2H, m), 2.88 p.p.m. (2H, t, $J = 6.3$ Hz), 3.32 p.p.m. (2H, m), 6.68 p.p.m. (2H, d, $J = 8.0$ Hz), 7.31 p.p.m. (2H, d, $J = 8.0$ Hz).

Conjugation of NPrCAP-SH with magnetite

To prepare the aminosilane-coated magnetite nanoparticles, 10 ml (concentration, 40 mg ml^{-1}) of magnetite nanoparticles and 0.1 ml of 3-aminopropyltriethoxysilane were mixed and incubated for 1 hour with stirring at room temperature. The resultant magnetic suspension was then washed three times with 10 mM phosphate buffer (pH 6.8) by centrifugation at 2,500 r.p.m. for 2 minutes and resuspended in 10 ml of phosphate buffer. For conjugation of maleimide cross-linkers, the magnetite suspension was mixed with 200 ml of *N*-[γ-maleimidobutyryloxy]sulfosuccinimide ester (10 mg ml^{-1}), and incubated in PBS for 30 minutes with shaking at room temperature. The resultant magnetite suspension was washed three times with water by centrifugation at 2,500 r.p.m. for 2 minutes. Then 0.5 ml of NPrCAP-SH (50.0 mg ml^{-1} of ethanol) was added to 10 ml of the magnetite suspension (40.0 mg ml^{-1}) and the mixture was stirred for 30 minutes at room temperature. After standing for 2 hours at room temperature, the suspension was washed twice with water by centrifugation at 3,000 r.p.m. for 1 minute. The resultant NPrCAP/M was resuspended in Milli-Q water to a concentration of 40 mg ml^{-1}.

Analysis of NPrCAP incorporated with magnetite nanoparticles

The degree of incorporation of NPrCAP-SH with magnetite was determined by hydrolysis with 6 M HCl followed by HPLC analysis of the resultant 4-*S*-CAP. Briefly, the amount of 4-*S*-CAP produced by the hydrolysis of NPrCAP/M with 6 M HCl at 110°C for 1.5 hours was measured by HPLC using 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 × 250 mm; 5.0 μm particle size). The UV detector was set at 250 nm. The mobile phase used was methanol:H_2O:1.0 M $HClO_4$, 10:90:1.5 by volume. The analyses were performed at 50°C at a flow rate of 0.7 ml minute^{-1}. The concentration of iron, which formed a red complex with thiocyanate, was quantitated by absorbance at 480 nm (Owen and Sykes, 1984).

Tyrosinase oxidation of NPrCAP/M

Mushroom tyrosinase (80 μg) was added to a reaction mixture containing 100 μM NPrCAP/M and 200 μM cysteine in 1 ml of 50 mM sodium phosphate buffer (pH 6.8), and oxidation was carried out at 37°C. At 30 minutes, 1 hour, and 3 hours, a 100-μl aliquot of the reaction mixture was removed and mixed with 900 μl of 0.4 M

$HClO_4$. The concentrations of the substrate remaining as 4-*S*-CAP and 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol produced were measured by hydrolysis with 6 M HCl followed by HPLC analysis (see above section, Analysis of NPrCAP incorporated with magnetic particles). The retention times of 4-*S*-CAP and 5-*S*-cysteaminyl-3-*S'*-cysteinylcatechol were 14.4 and 7.9 minutes, respectively.

Measurement of iron in the NPrCAP/M-exposed cells

Subconfluently growing melanoma and non-melanoma cells (8×10^4 cm^{-2}) in a 25 cm^2 flask were refed with the medium containing 5.94 mg of NPrCAP/M or magnetite (84 μg ml^{-1}). To discriminate between incorporation of NPrCAP/M by direct attachment to cells and that by diffusion from the medium, culture flasks were fixed on a slanted disc (60°) and rotated slowly for 30 minutes. After the cells were washed with PBS twice and collected, they were dissolved completely in 200 μl of concentrated HCl and incubated at 43°C for 30 minutes. Then, 10 μl of H_2O_2 and 4 ml of 1% potassium thiocyanate were added in sequence to the cell solution. The iron concentration of magnetite nanoparticles was measured using the potassium thiocyanate method as described above.

Cytotoxicity of NPrCAP/M in combination with AMF irradiation

For cytotoxicity measurement, 5×10^6 B16F1, MM418, SK-mel-23, TXM18, H1229, HaCaT, HeLa, and SaOS2 cells were cultured in the medium containing 5.94 mg of NPrCAP/M or magnetite (1.19 mg ml^{-1}) for 20 minutes. Then, the cells were harvested by centrifugation at 400 *g* for 10 minutes and the cell pellets were subjected to AMF irradiation at 43°C for 30 minutes. The AMF was generated by using a horizontal coil (inner diameter 7 cm, length 7 cm) with a transistor inverter (LTD-100-05; Dai-ichi High Frequency Co., Tokyo). The magnetic field frequency and intensity were 118 kHz and 30.6 kA/M (384 Oe), respectively. Cell temperatures were measured and monitored using an optical fiber probe (Anritsu Meter, Tokyo, Japan). After AMF irradiation, aliquots (1/10) of cells were seeded in a dish and cultured for a further 48 hours. Viable cells were counted using a hemocytometer.

Recombinant adenoviruses

Propagation, plaque formation, and inoculation of recombinant adenoviruses were described elsewhere (Yamano *et al.*, 1999). Ad-LacZ is a replication-deficient recombinant adenovirus carrying β-galactosidase. Ad-p63′ is a recombinant adenovirus expressing modified p63, which was provided by T Tokino (manuscript in preparation and personal communication). The construction of the original adenovirus Ad-p63 containing the human p63 gene was described previously (Sasaki *et al.*, 2001, 2003). Cells were infected with 20 plaque-forming units of a recombinant adenovirus, incubated for 60 minutes at 37°C, and cultured in fresh DMEM with 5% FBS for 48 hours before flow cytometric analysis or caspase assay.

Flow cytometric analysis

After B16F1 and SK-mel-23 cells were cultured in the NPrCAP/M-containing medium (4.6 mg ml^{-1}) for 30 minutes, they were subjected to AMF irradiation at 43°C for 30 minutes, reseeded in the medium, and cultured for 24 hours. Then adherent and floating cells were collected together and washed in ice-cold PBS. The cells were dehydrated in 75% cold ethanol and stored on ice for 2 hours. Then they were rehydrated in cold PBS and incubated in the presence of RNaseA (50 μg ml^{-1}) (Sigma Aldrich Japan, Tokyo, Japan) at 37°C for 30 minutes. After incubation, the cells were rinsed twice in ice-cold PBS and suspended in 2.0 ml of PBS containing 50 μg ml^{-1} propidium iodide (Sigma Aldrich Japan) at 4°C for 2 hours. Cell debris and fixation artifacts were gated out, and sub-G1, G1, S, and G2/M populations were quantified with a FACScan Cell Sorter (Nippon Becton Dickinson, Tokyo, Japan) using the CELL QUEST program.

Caspase enzyme assay

After 5×10^6 B16F1 and SK-mel-23 cells were collected in 15 ml tubes, 5.94 mg of NPrCAP/M was separately added to the cell pellets, which were then incubated for 20 minutes at 37°C in a CO_2 incubator. Cells were harvested by centrifugation at 400 *g* for 10 minutes and irradiated by the AMF as described above. Cells were then collected and seeded in 10 cm dishes and cultured for a further 24 hours. Both the floating and the adherent cells were collected, washed with PBS, and processed for caspase assay. Activities of caspases 3, 8, and 9 were measured using a colorimetric protease kit according to the manufacturer's protocol (MBL, Nagoya, Japan).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by a Health and Labor Sciences Research Grant-in-Aid for Research on Advanced Medical Technology from the Ministry of Health, Labor, and Welfare of Japan. We thank T Moriuchi of Hokkaido University, Sapporo, Japan, for providing us with MMIV and AK-1 melanoma cells. We also thank K Enomoto of Akita University for providing us with HaCaT cells and M Ojika of Nagoya University, Graduate School of Bioagricultural Sciences, Nagoya, Japan, for the measurement of ^{1}H NMR. We thank Toda Kogyo Co. for supplying the magnetite.

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