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Cancer Science
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Human leukocyte antigen class I down-regulation in muscle-invasive bladder cancer: Its association with clinical characteristics and survival after cystectomy

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Using a novel monoclonal anti-pan human leukocyte antigen (HLA) class I heavy chain antibody (EMR 8-5) reacting with paraffin-embedded sections, we examined the prognostic significance of HLA class I molecules in muscle-invasive bladder cancer patients who underwent radical cystectomy. Immunohistochemical staining for HLA class I molecules with monoclonal antibody EMR 8-5 was performed on specimens from 65 clinically muscle-invasive bladder cancer patients who underwent radical cystectomy and pelvic lymph node dissection without neoadjuvant chemotherapy. We analyzed the clinicopathological and prognostic significance of HLA class I expression. Immunohistochemical analysis revealed HLA class I down-regulation in 22 (33.8%) invasive bladder cancers. This down-regulation had no correlation with clinicopathological parameters such as pathologic stage, nodal status, and grade. The recurrence-free survival of patients with HLA class I-positive tumors was significantly better than that of those with down-regulation (log rank, *P* = 0.0337). Multivariate analysis revealed that HLA class I expression was a significant factor influencing the recurrence-free survival of bladder cancer patients after cystectomy (*P* = 0.0155). Our data demonstrate that HLA class I down-regulation in tumor cells was clearly observed in about one-third of the patients. HLA class I expression could be a prognostic marker for muscle-invasive bladder cancer patients after cystectomy. (*Cancer Sci* 2009; 100: 2331–2334)

Radical cystectomy with pelvic lymph node dissection is a standard surgical procedure for muscle-invasive bladder cancer, with a 5-year survival rate of approximately 60%. However, one-third of the patients treated by cystectomy die of the disease, mostly of metastatic spread. Pathologic stage and nodal involvement are reported to be the most important prognostic factors in bladder cancer.[1] However, there are few other clinicopathological parameters predicting the survival of bladder cancer patients after cystectomy.

Human leukocyte antigen (HLA) class I molecules have a central role in the cell-mediated immune system, especially as antigen-presenting molecules for cytotoxic T lymphocytes (CTLs). CTL can recognize antigenic peptides presented on the cell surface with HLA class I molecules, and kill the target cell.[2,3] Down-regulation of HLA class I was found to be implicated in the immune escape of malignant tumors.[2,3] It is reported that this phenomenon is observed in malignant tumors such as malignant melanoma, colorectal, lung, and ovarian cancers,[4–7] and affects survival of a limited number of patients with these diseases.

We have recently established a novel anti-pan HLA class I heavy chain mAb, EMR8-5, which can detect HLA-A, B, and C antigens in formalin-fixed paraffin-embedded tissue sections.[8–10] Using this mAb, we previously reported that HLA class I down-regulation was observed in about one-third of superficial bladder cancers and that HLA class I expression contributed significantly to the therapeutic effect of Bacillus Calmette-Guérin (BCG) immunotherapy.[9]

In this study, we immunohistochemically examined the expression profiles of HLA class I molecules in patients with muscle-invasive bladder cancer who underwent radical cystectomy, and analyzed the prognostic significance of the expression.

Materials and Methods

Patients and tissues. We reviewed the clinical pathology archives of consecutive patients with muscle-invasive bladder cancer who underwent radical cystectomy and regional pelvic lymph node dissection without neoadjuvant chemotherapy from January 1991 to December 2002 at Sapporo Medical University Hospital. The study protocol was approved by the Clinical Institutional Ethical Review Board of the Medical Institute of Bioregulation, Sapporo Medical University, Japan. Bladder cancer was histopathologically diagnosed by transurethral resection (TUR) in all patients before cystectomy. No patients had distant metastasis at the time of the initial diagnosis. Radical cystectomy and regional pelvic lymph node dissection were performed using a standard technique.[11] Pelvic lymph node dissection included the internal iliac, external iliac, and obturator lymph nodes. Boundaries of dissection included the circumflex iliac vein inferiorly, pelvic side wall laterally, bladder wall medially and common iliac bifurcation superiorly. Concurrent urethrectomy was performed for male patients with a histologically proven cancer on the prostatic urethra. Anterior pelvic exenteration was done in women who selected urinary diversion other than an orthotopic ileal neobladder.

We finally selected 65 patients, based on the availability of sufficient material for immunohistochemical evaluation, before obtaining information on their clinical outcomes. All hematoxylin–eosin-stained slides were reviewed, and clinical stage was assigned using the 1997 TNM classification[12] and the World Health Organization system.[13]

Immunohistochemistry by EMR 8-5. Immunohistochemical staining with the monoclonal anti-pan HLA class I (HLA-A, B, and C) antibody EMR8-5, which was established at our laboratory, was performed as previously described.[14] Human tonsil sections were used as positive controls for HLA class I. Staining of vascular endothelial cells and lymphocytes in sections were used as an internal positive control for immunostaining. Negative controls were done by omitting the primary antibody. All specimens were reviewed independently using light microscopy in at least five areas at ×400 magnification by investigators who were blinded to clinicopathological data (TT). The membrane

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immunoreactivity level for HLA class I was categorized from undetectable to +2. A score of zero was defined as undetectable staining. A score of +1 was defined as faint, incomplete membrane staining in more than 20% of the tumor cells, or as moderate to complete staining in cytoplasm but negative membrane staining in the tumor cells (Fig. 1a,b). Finally, a score of +2 was defined as complete membrane staining in more than 80% of the tumor cells (Fig. 1c,d). HLA class I expression was then classified as down-regulated (scores 0 and 1) or positive (score 2).

Statistical analysis. Differences in clinicopathological characteristics between HLA class I positive and down-regulated tissues were assessed using the Mann–Whitney test. Survival time was analyzed from the date of surgery. The end points of univariate and multivariate analyses were recurrence-free survival. Survival estimates were constructed using the Kaplan–Meier method. The log-rank test was used to evaluate the significance of differences in the univariate analysis. For multivariate analysis, Cox's proportional hazards model was used. A value of $P < 0.05$ was considered to indicate statistical significance.

Results

Of the 65 patients, 51 were men and 14 were women. They ranged in age from 38 to 79 years (mean, 65.2 years). Clinically, 42 patients (64.6%) were diagnosed as having extravesical disease (T3 or more) before cystectomy (Table 1). Preoperative computed tomography demonstrated suspected metastasis to the pelvic lymph nodes in five patients. Pathologically, 28 patients (43.1%) had tumors confined to the bladder (pT2 or less) and 33 patients (50.8%) had tumors penetrating the bladder wall into perivesical fat or adjacent structures (pT3 or more). Pathological pelvic lymph node metastasis was found in 11 patients (16.9%). Histology of pure urothelial carcinoma was found in 49 patients (75.4%) and other histological components such as squamous cell carcinoma and adenocarcinoma in 16 (24.6%). Adjuvant chemotherapy was done for five patients (7.7%) with pathological T3 or T4 disease and/or nodal involvement according to the preferences of both the patients and urologists.

As shown in Table 1, of the 65 bladder cancer specimens, 43 (66.2%) were graded as having high expression of HLA class I molecules. HLA class I down-regulation was found in 22 specimens (33.8%), with 19 having low and three having negative expression. In organ-confined disease (pathological T2 or less disease with negative nodal involvement) and extravesical disease (pathological T3 or T4 disease and/or nodal involvement), HLA class I down-regulation was observed in 10 specimens (35.7%) of 28 and 12 specimens (32.4%) of 37, respectively. There was no correlation between HLA class I down-regulation and pathological stage. Moreover the status of HLA class I expression had no correlation with other clinicopathological parameters such as clinical stage, nodal status, histology, and grade (Table 1).

The median follow-up period of the 65 patients was 35 months, ranging from 3 to 172 months. The median follow-up of the 37 survivors was 52.5 months. Distant metastases and/or local recurrence developed in 33 of the 65 patients (50.8%) at a median of 11 months (range, 3–74 months) after surgery. The 5-year recurrence-free survival was 46.0% for all patients. The 5-year survivals were 55.0% and 30.3% in the HLA class I positive and down-regulated arms, respectively. Patients with HLA class I positive expression had significantly longer recurrence-free survival than those with down-regulated expression (log rank, $P = 0.0337$) (Fig. 2a). Although there was no significance because of the small number of patients, in extravesical disease (pathological T3 or T4 disease and/or nodal involvement), in particular, HLA class I expression seemed to

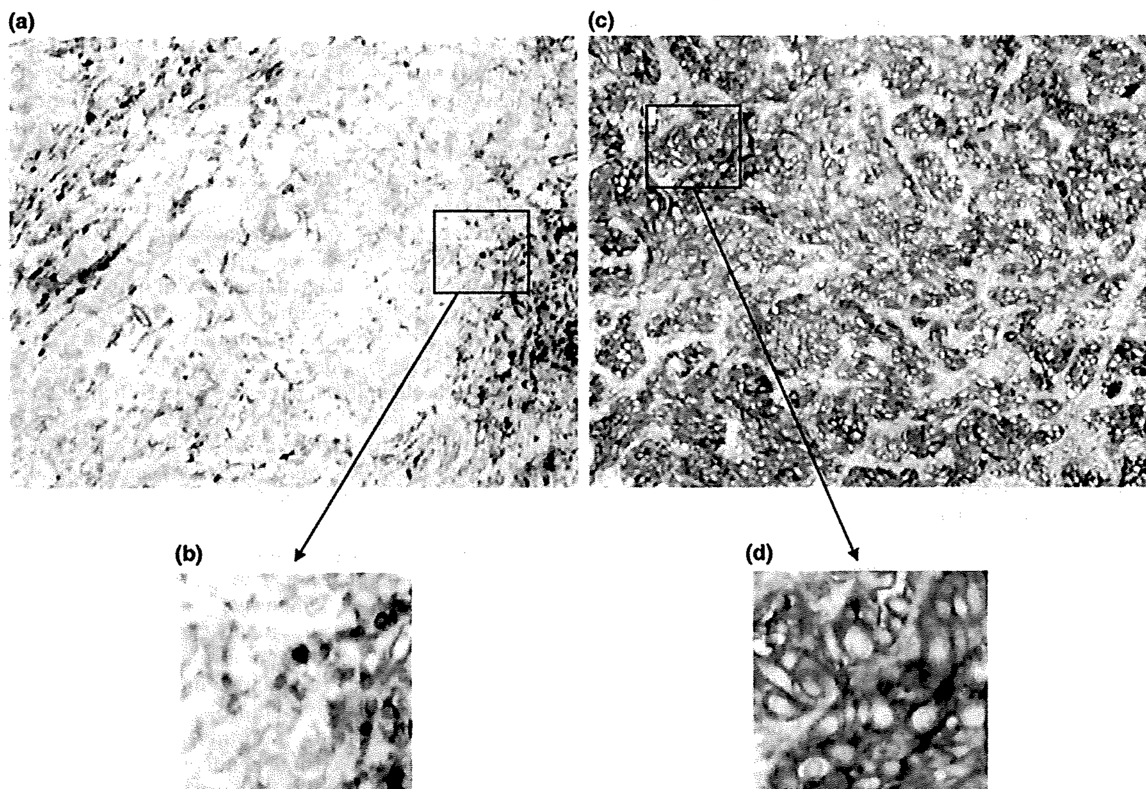

Fig. 1. Representative pictures of immunohistochemical staining with mAb EMR 8-5 reacting to human leukocyte antigen (HLA) class I molecules in invasive bladder cancer. (a) Stained tumor cells are seen in cytoplasm but not in the cell membrane, demonstrating down-regulation of HLA class I molecules. (b) Magnified view of the box in panel A. (c) Cytoplasmic portion and cell membranes of tumor cells are also completely stained. (d) magnified view of the box in panel C.

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Table 1. Clinical characteristics, pathological and nodal status, and tumor histology

Clinical and pathological characteristics	HLA class I positive	HLA class I down-regulation	*P*-values
No. of patients	43	22	
Clinical stage			
cT2N0	18 (40.0)	5 (26.1)	0.1188
cT3N0	14 (33.3)	8 (34.8)	
cT4N0	8 (20.0)	7 (30.4)	
cT2-4N+	3 (6.7)	2 (8.7)	
Pathological stage			
pT1-2N0	18 (42.3)	10 (43.5)	0.8837
pT3N0	11 (24.4)	4 (21.7)	
pT4N0	8 (20.0)	3 (13.1)	
pT1-4N+	6 (13.3)	5 (21.7)	
Histology			
Pure UC	32 (73.3)	17 (73.9)	0.8020
Aberrant differentiation with or without UC	11 (26.7)	5 (26.1)	
Grade			
1–2	10 (20.0)	7 (30.4)	0.4608
3	33 (80.0)	15 (69.6)	

HLA, human leukocyte antigen; UC, urothelial carcinoma.

be an important factor for predicting prognosis (Fig. 2b,c). As shown in Table 2, multivariate analysis revealed that, in addition to pathological stage, HLA class I expression was a significant factor influencing the disease-free survival of bladder cancer patients after cystectomy ($P = 0.0155$).

Discussions

Down-regulation of HLA class I expression has been demonstrated in various types of solid tumors and is considered to be one of the mechanisms of tumor immune escape.[2,3] Some studies have reported that this down-regulation could be a significant prognostic factor for various malignant tumors.[4–7] An anti-HLA class I mAb detecting HLA antigens in formalin-fixed paraffin-embedded histological biopsy and surgically resected tissue specimens would be highly useful in determining the exact characteristics of the HLA class I expression in tumors. As such mAb HC-10 which reacted with HLA-B and C alleles was established.[15]

We have recently established a novel monoclonal anti-pan HLA class I heavy chain antibody, EMR8-5, which can detect HLA-A, B, and C antigens in paraffin-embedded sections.[8–10] Using this monoclonal antibody, we examined the expression profiles of HLA class I molecules immunohistochemically in muscle-invasive bladder cancer, and analyzed the prognostic significance of HLA class I expression.

Previous studies of HLA class I antigen expression in bladder cancers have reported that HLA class I down-regulation was observed in between 27% and 44% of patients,[16–18] results similar to those observed in our previous study.[9,14] In this study HLA class I down-regulation was observed in 33.8% of muscle-invasive bladder cancers.

We found that HLA class I antigen down-regulation affected survival in invasive bladder cancer patients. In contrast, some studies of malignant melanoma have shown a lack of prognostic significance for HLA class I expression.[19,20] It should also be noted that total loss of HLA class I has been proposed as an indicator of good prognosis in breast cancer and non-small-cell lung cancer.[21,22] In those reports, the authors mentioned that HLA class I antigen down-regulation may make the tumors more susceptible to natural killer (NK) cell killing and result in a better prognosis. Our preliminary data from immunohistochemical staining using monoclonal antibodies for cluster of differentiation (CD)-8 and CD56 seemed to indicate that infiltration of CD8-positive cells was increased in HLA class I

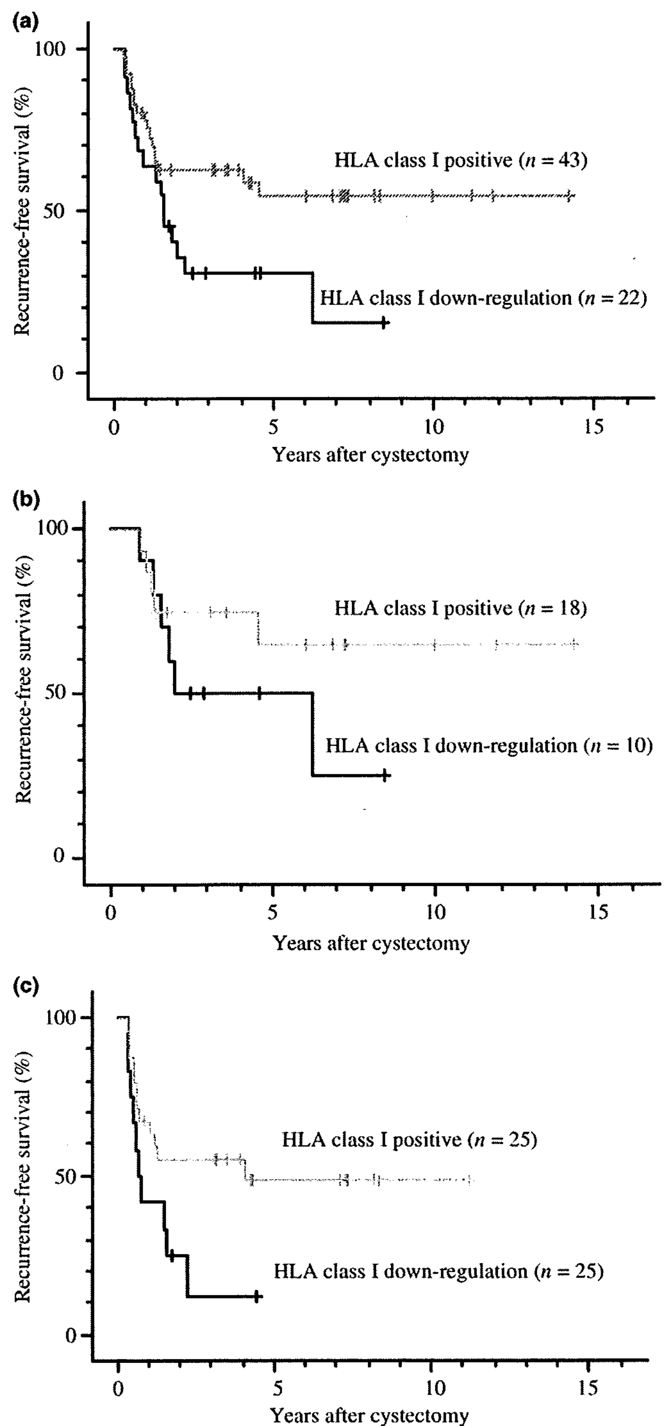

Fig. 2. Recurrence-free survival 65 patients stratified by HLA class I expression. (a) Comparison of HLA class I positive findings ($n = 43$) and HLA class I down-regulation ($n = 22$) in all 65 patients ($P = 0.0337$). (b) Comparison of HLA class I positive findings ($n = 18$) and HLA class I down-regulation ($n = 10$) in 28 patients with pT1-2 node-negative disease ($P = 0.2274$). (c) Comparison of HLA class I positive findings ($n = 25$) and HLA class I down-regulation ($n = 12$) in 39 patients with pT3-4 and/or node-positive disease ($P = 0.0566$). All *P*-values were determined by the log-rank test.

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Table 2. Univariate and multivariate analyses of parameters predicting disease-specific survival

Factor	Univariate *P*-values	Multivariate *P*-values	Hazard ratio	95% CI
Histology: pure UC *versus* aberrant differentiation	0.9696	0.8981	1.052	0.484–2.285
Pathological stage: pT1-2 *versus* pT3-4	0.0095	0.0093	2.604	1.266–5.358
Nodal involvement: no *versus* yes	0.2053	0.1364	1.947	0.810–4.679
HLA class I: positive *versus* down-regulation	0.0337	0.0155	2.392	1.180–4.848

CI, confidence interval; HLA, human leukocyte antigen; UC, urothelial carcinoma.

positive cases (data not shown). This implied that the HLA class I-restricted CTLs were mobilized around HLA class I–positive cancer cells. However, CD56-positive cells were rarely observed in either HLA class I–positive or down-regulated cases. This finding suggested that the immune surveillance system via the HLA class I–restricted CTL pathway may play a role in the regulation of microscopic metastatic disease progression in advanced extravesical disease, in particular. However, NK cells might not act for tumor rejection.

In recent years there have been many studies of specific immunotherapies for various cancers.[23,24] The rationale for such studies has been supported by strong cellular immune responses, that is introducing cancer-specific CTLs from patients. The effectiveness of immunotherapy using tumor-specific antigens largely depends on the expression of the appropriate HLA class I alleles on the tumor cells. In the future, when T cell-based immunotherapy is available for the treatment of bladder cancer, it will be necessary to evaluate and clarify the defects in the antigen-presentation machinery that are likely to generate cancer cells able to escape the host's T-cell control.

In this study, for accurate pathological evaluation, we excluded patients with pT0 and those who received neoadjuvant chemotherapy. Moreover, patients whose formalin-fixed paraffin-embedded tissue was incomplete were excluded. In this limited patient group with more high-stage disease, nodal status did not affect the prognosis after cystectomy. Therefore this study may have some limitations; however, the results of the current study suggest that HLA class I expression may be one of the important prognostic factors in advanced extravesical disease, in particular. Moreover HLA class I expression may be one of the important factors to select proper patients for treatment with CTL-based immunotherapy in the future. Further studies are required to elucidate the mechanisms of HLA class I gene down-regulation and up-regulation.

Conclusions

Our data demonstrated that HLA class I down-regulation on tumor cells could be observed in about one-third of patients and that it was an independent prognostic factor for muscle-invasive bladder cancer, especially in patients with extravesical disease. This finding suggests that HLA class I expression could be a prognostic marker in muscle-invasive bladder cancer patients after cystectomy.

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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.

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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*

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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).

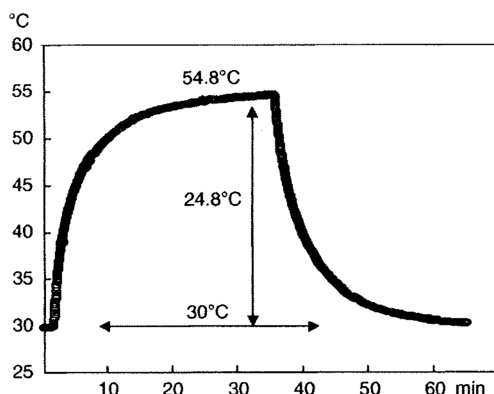

Figure 1. Heat generation in cells treated with NPrCAP/M and irradiated by AMF. 2 × 10^6 B16F1 cells were cultured in NPrCAP/M (5.0 mg magnetite equivalent)-containing medium for 30 minutes, collected, and exposed to the center of the coil of the AMF generator. The temperature at the center of the cell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

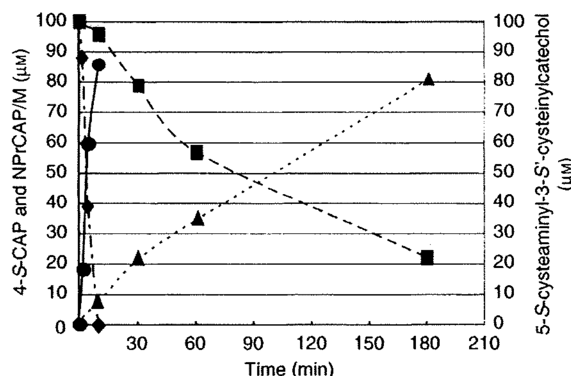

Figure 2. NPrCAP/M is incorporated into the tyrosinase oxidative reaction *in vitro*. The concentrations of the substrate remaining as 4-*S*-CAP and the 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol produced were measured by HPLC analysis after hydrolysis with HCl. ◆: 4-*S*-CAP, ■: NPrCAP/M, ●: 5-*S*-cysteaminyl-3-*S*′-cysteinylcatechol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

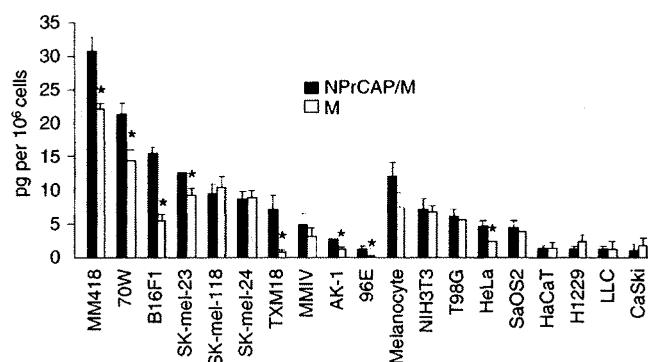

Figure 3. Uptake of magnetite nanoparticles into melanoma and non-melanoma cells. 75 cm^2 flasks containing growing cells were filled with NPrCAP/M- or magnetite-containing medium and fixed on a slanted disc, which was rotated slowly for 30 minutes. Incorporated iron was measured by the potassium thiocyanate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

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

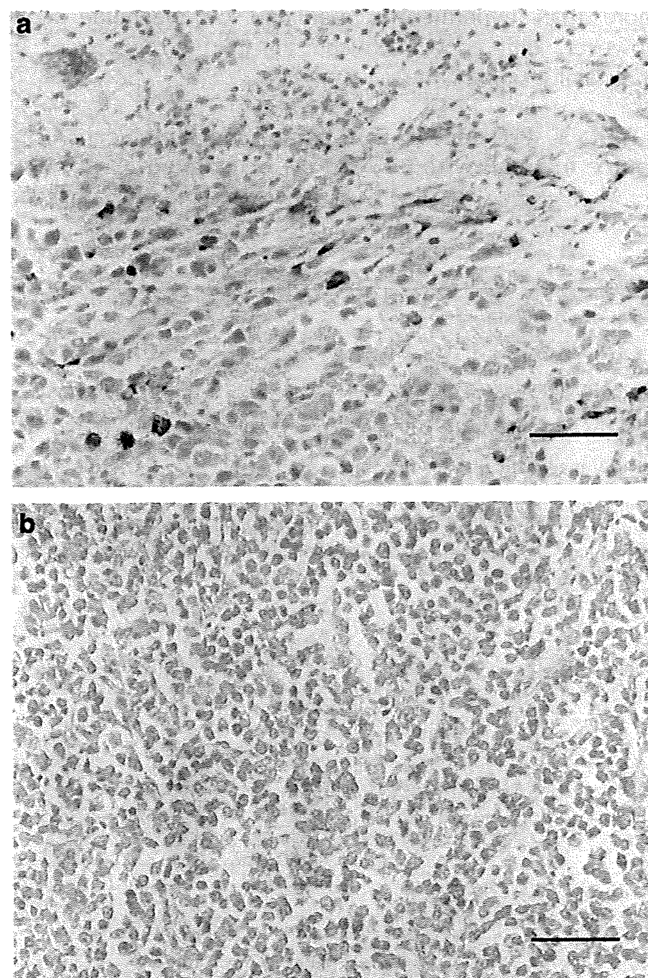

Figure 4. Intraperitoneal NPrCAP/M nanoparticles were delivered to the subcutaneously transplanted melanoma tumors. Mice bearing B16F1 and lymphoma tumors received i.p. administration of NPrCAP/M and were maintained for 14 days. Tumors were then removed and processed for hematoxylin-eosin and Berlin 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**).

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;

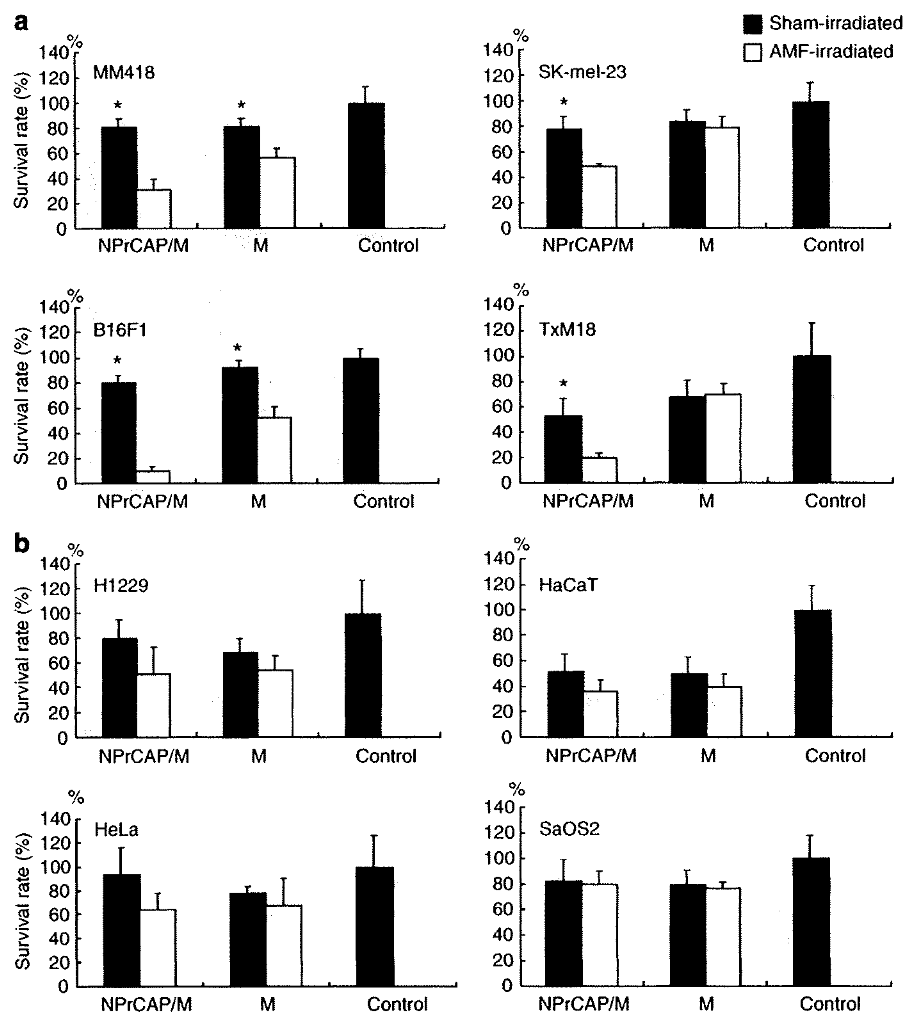

Figure 5. NPrCAP/M plus AMF treatment degraded melanoma cells more significantly than NPrCAP/M without the AMF. After cells were cultured in the NPrCAP/M-containing medium and collected, cell pellets were exposed to sham (■) or AMF (□) irradiation. Treated cells were collected and the number of viable 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.

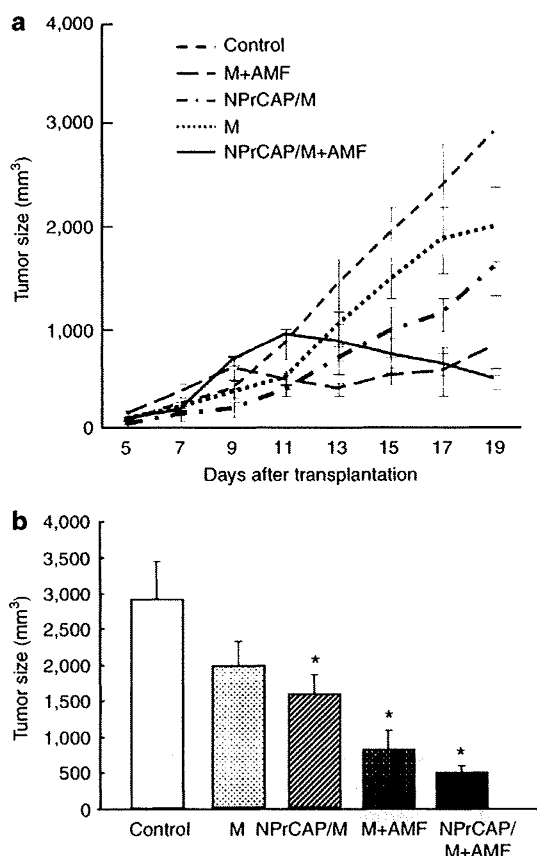

Figure 6. Tumor volumes of the B16F1-bearing mice treated by magnetite- and NPrCAP/M-mediated hyperthermia. (**a**) Comparison of groups in the first 19 days after tumor transplantation. Magnetite or NPrCAP/M (4 mg of magnetite or its equivalent) was injected directly into subcutaneous B16F1 tumors, 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.

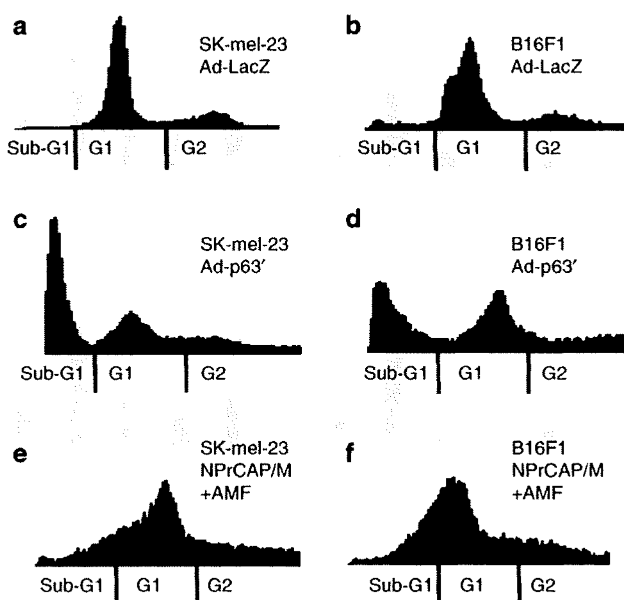

Figure 7. The sub-G1 fraction was not observed in NPrCAP/M-treated and AMF exposed cells. After cells were treated with NPrCAP/M followed by AMF irradiation and culture for 24 or 48 hours, adherent and floating cells were collected and sub-G1, G1, S, and G2/M populations were quantified with a FACScan 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 cysteaminylphenols 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

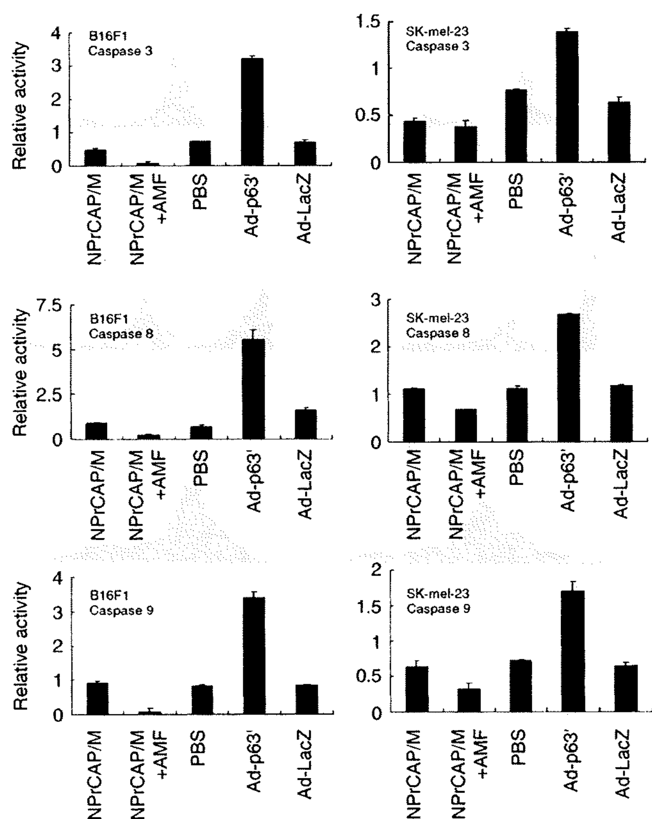

Figure 8. Caspases 3, 8, and 9 were not activated in cells that received NPrCAP/M-mediated hyperthermia. B16F1 mouse melanoma cells and SK-mel-23 human melanoma cells were treated with NPrCAP/M with or without hyperthermia. Cells were infected with 20 pfu per cell of Ad-p63′ or Ad-LacZ for 24 hours for 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-Aminopropyltriethoxysilane 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. ^1H 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 ($8 \times 10^4\,cm^{-2}$) in a 25 cm^2 flask were refed with the medium containing 5.94 mg of NPrCAP/M or magnetite ($84\,\mu g\,ml^{-1}$). 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 ($50\,\mu g\,ml^{-1}$) (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 $50\,\mu g\,ml^{-1}$ 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.

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Establishment of shared antigen reactive cytotoxic T lymphocyte using co-stimulatory molecule introduced autologous cancer cells

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abstract

Cytotoxic T lymphocytes (CTLs) play an essential role in immunological responses for tumor rejection. In the past decade, many tumor-associated antigens (TAAs) have been identi ed predominantly in melanomas. Several clinical trials based on such antigenic peptides with or without adjuvants brought about partially favorable results, suggesting that identi cation of more immunogenic TAAs is needed. We show here the successful establishment of human leukocyte antigen (HLA)-A24-restricted CTL (TcLHK2 line1) from a pleural effusion of lung cancer patient, using B7.1 (CD80) transduced autologous lung cancer cells as an antigen-presenting cell (APC). TcLHK2 line1 recognized autologous lung adenocarcinoma cell line LHK2 in an HLA-A24-restricted fashion. Moreover, this CTL line also recognized allogeneic HLA-A24-positive lung adenocarcinoma cell line, gastric carcinoma cell line and melanoma cell line. These data raise the possibility that co-stimulatory molecule B7.1 (CD80) plays important role to overcome the immunological tolerance. Furthermore, TcLHK2 line1 is a useful tool for the identi cation of widely expressed shared antigens restricted by HLA-A24. Further analysis of this CTL and autologous cancer cell line will bring about novel TAAs.
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Introduction

Recently, huge amounts of TAAs have been identi ed. Although some of them are expressed in various types of tumor cells, most have been characterized with anti-tumor CTLs isolated from the peripheral blood mononuclear cell (PBMC) of melanoma patients (Boon et al., 1997; Rosenberg, 1999). However, little is known about TAAs recognized by autologous CTLs in lung cancer because of the dif culties in the generation of tumor-speci c CTLs. Possible major reasons for the dif culties generating CTLs for lung cancer cells in vitro might be the insuf ciency of the expression of TAAs or co-stimulatory molecules on tumor cells. Some lung cancer expressed that TAAs have been described previously by reverse-immunogenetical approach (Hirohashi et al., 2009), however, analysis of naturally expressed TAAs is essential for the identi cation of more immunogenic antigens.

It is now well accepted that T cells require at least two signals for their full activation. The rst signal is delivered through interaction of a T cell receptor with an MHC antigenic peptide complex. The second signal is brought about interaction of receptors on T cells (e.g. CD2, CD40-L, LFA-1 or CD28) and co-stimulatory molecules on antigen-presenting cells (e.g. LFA-3, CD40, ICAM-1, CD80 or CD86). The absence of the second signal leads to clonal inactivation (Schwartz, 1990) or activation-induced cell death of T cells (Liu and Janeway, 1990), and this mechanism is thought to be related to anergy or immunological tolerance. In animal models, the co-stimulation mediated by B7.1 (CD80) plays an important role in the induction of T cell-mediated anti-tumor immunity (Chen et al., 1992; Townsend and Allison, 1993). In human malignancies, a few studies have been performed to assess the effect of B7.1 (CD80) on the induction of tumor-speci c CTLs from patients with melanoma (Yang et al., 1997), cervical carcinoma (Kaufmann et al., 1996), ovarian cancer (Gilligan et al., 1998) and colon cancer (Miyazono et al., 1999).

We previously reported human gastric carcinoma (Yasoshima et al., 1995), pancreas carcinoma (Ueda et al., 1995; Kashiwagi et al., 2003), head and neck carcinoma (Miyazaki et al., 1997; Kobayashi et al., 2009), and osteosarcoma (Nabeta et al., 2003) speci c autologous CTLs derived from peripheral blood or abdominal cavities. However, it still remains dif cult to induce autologous tumor-speci c CTLs in patients with lung cancer. We show here the successful generation of

Abbreviations: CTLs, cytotoxic T lymphocytes; TAAs, tumor-associated antigens; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; MLTC, mixed lymphocyte tumor cell culture; TILs, tumor in ltrating lymphocytes; mAb, monoclonal antibody; FCM, owcytometer.

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autologous tumor-speci c CTL line from patients with lung adenocarcinoma, using B7.1 (CD80) transduced tumor cells as APCs. This CTL line, termed as TcLHK2 line1, recognized autologous adenocarcinoma cell line (LHK2) in an HLA-A24-restricted fashion. Moreover, these CTLs could also recognize allogeneic lung adenocarcinoma cell line, gastric signet ring cell carcinoma cell line and melanoma cell line. These ndings indicate that TcLHK2 line1 recognized tumor-shared antigens that is expressed in variety of histology, and further analysis of this CTL line will bring immunogenic TAA.

Materials and methods

Cell lines

LHK2, lung poorly differentiated adenocarcinoma cell line, was established from pleural effusion of 68-year-old male lung cancer patient who had died of his disease 3 weeks later, and, cultured in RPMI 1640 medium (SIGMA, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Palo Alto, CA), L-glutamine (Invitrogen) and antibiotics. Patients HLA genotypes were con rmed in our laboratory as HLA-A 0207, A 2402, B 4601, B 4801, Cw 0102. Autologous TILs (tumor in ltrating lymphocytes) were also established from patient's pleural effusion, and cultured in AIM-V medium (Invitrogen) supplemented with interleukin (IL)-2 1000 international unit (IU)/ml for 3 weeks and frozen in -80 °C for stock. Lung adenocarcinoma cell line, LNY1 (HLA-A26, A31, B51, B54, C4, C10), stomach signet ring cell carcinoma cell line, SSTW (HLA-A 2402, A 2601, B 4601, B 1501, Cw 0102, Cw 0303), and osteosarcoma cell line, OS2000 (HLA-A24, B55, B61, Cw1) were established in our laboratory previously. Anti-HLA-A2, A28 mAb hybridoma, PA2.1, anti-HLA-A11 and A24 hybridoma, A11.1M, erythroleukemia cell line K562 and gastric signet ring cell carcinoma, KATOIII (HLA-A2, A 2402, B 1501, B 4601, Cw 0102, Cw 0303) were purchased from the America Type Culture Collection (ATCC). A melanoma cell line, LG2-mel, a bladder cancer cell line, LB905-BLC, a sarcoma cell line, LB23-SARC, anti-HLA-B,C hybridoma B1.23.2, and LG2-EBV EBV-transformed B cell line were kind gifts from Dr. P. G. Coulie (Ludwig Institute for Cancer Research, Brussels Branch, Brussels, Belgium). A melanoma cell line 1353-mel was a kind gift from Dr. F. M. Marincola (National Cancer Institute, Bethesda, MA). Pancreas adenocarcinoma cell line, Panc-1 was a kind gift from Dr. K. Imai. (Sapporo, Japan). PC-9 lung adenocarcinoma cell line and KE-4 esophagus squamous cell carcinoma cell line were kind gifts from Dr. K. Ito (Kurume, Japan). All these cells were cultured in RPMI 1640 media supplemented with 10% FBS and L-glutamine.

Flowcytometer (FCM) analysis and monoclonal antibodies (mAbs)

Cells were incubated with mouse mAbs at saturating concentration for 30 min on ice, washed with PBS and stained with polyclonal goat anti-mouse antibody coupled with FITC for 30 min. Samples were analyzed by FACSCalibur analyzer (Becton Dickinson, Mountain View, CA). Anti-pan HLA class I mAb (W6/32) and anti-HLA-DR mAb (L243) were prepared from hybridomas. Anti-B7.1 (CD80) mAb (MAB104) was purchased from Immunotech (Marseille, France).

Cloning of HLA cDNA

Total RNA from LHK2 lung adenocarcinoma cell line was extracted with ISOGEN reagent according to the manufacturer's protocol (Nippon gene, Tokyo, Japan), and was reverse-transcribed by using Superscript II reverse transcriptase with oligo (dT) primer (Invitrogen). The incubation was carried out at 42 °C for 60 min and then at 70 °C for 15 min. PCR procedure with Pfu DNA polymelase was performed using the forward primer 5 -GACTCAGATGATATCCAGACGCCGAGGATGGCCGTCATG-3 and the reverse primer 5 -CGCGGATCCGCGGCCGCAGGGAGCACAGGTCAGCGTGGGAA-3 , which are speci c for HLA gene and contain EcoRV and BamHI restriction site, respectively. The mixture was denatured at 98 °C for 5 min, then, run for 30 cycles at 98 °C for 15 s, 58 °C for 45 s, and 72 °C for 4 min. Puri ed PCR products were digested with EcoRV and BamHI, and, subcloned into pIRESpuro mammalian expression vector (Clontech, Palo Alto, CA). The insert was sequenced by ABI Genetic analyzer PRIM 310 using AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA).

Transfection

A mammalian expression vector pBJ-B7.1 which includes the human B7.1 (CD80) gene was a kind gift from Dr. M. Azuma (Tokyo, Japan). Transfection with Lipofectin reagent (Invitrogen) was performed following the manufacturer's procedure. Brie y, 24 h before transfection 3×10^5 tumor cells were plated in 6 well plate (Nunc). After 2 times of washing with serum-free medium, mixture of 2 g cDNA and 10 l Lipofectin in 2 ml OPTI-MEM medium (Invitrogen) was overlaid onto the cells. Six hours later, the culture media were replaced with the fresh one. Two days later, antibiotics for selection were added. For B7.1 (CD80) transfection, G418 (Invitrogen) was added at 500 g/ml, for HLA transfection, puromycin (Clontech) was added at 500 ng/ml. Antibiotics resistant cells were cloned and positive expressing clone was screened by FCM analysis.

Induction of CTL

Mixed lymphocytes tumor cell culture (MLTC) was performed as described previously (Yasoshima et al., 1995). Brie y, after thawing the TILs, $CD8^+$ T cells were positively separated with immunomagnetic beads (Invitrogen) following manufacturer's procedure, and 2×10^6 $CD8^+$ T cells were co-cultured in 24 well plate with 2×10^5 irradiated LHK2 or LHK2-B7.1 in 2 ml of AIM-V medium supplemented with 10% of human serum (HS). Three days later, human recombinant IL-2 (a kind gift from Takeda Pharmaceutical Co. Osaka, Japan) was added at nal concentration of 50 IU/ml. Same MLTC procedure was performed every 7 days. After 4 times of stimulation, cytotoxic activity was measured with standard ^{51}Cr release assay.

Rapid expansion method of CTL

To obtain suf cient number of cells for further analysis, CTLs were expanded in a similar method described by Maeda et al. (2001). 5×10^4 CTLs were resuspended in 25 ml of AIM-V medium supplemented with 10% HS, anti-CD3 Ab (40 ng/ml) with 2.5×10^7 allogeneic irradiated PBMCs (100 Gy) and 5×10^6 irradiated LG2-EBV (100 Gy). One day after initiating the culture, 150 IU/ml of IL-2 was added. The cultures were fed with fresh AIM-V supplemented with 10% HS and IL-2 50 IU/ml on day 5, 8 and 11. On day 14 expanded cells were assessed for their cytotoxic activities.

Cytotoxicity assay and blocking assay by mAbs

The cytotoxic activity of CTLs was tested by a ^{51}Cr release assay as described previously (Yasoshima et al., 1995). Brie y, 2000 target cells labeled with 100 Ci of chromium were incubated in 96-well micro-titer plates with CTLs at different E/T ratios in 200 l of AIM-V medium. ^{51}Cr release was measured after 6 h of incubation. For the inhibition assay, ^{51}Cr labeled target cells were incubated with mAbs of appropriate concentrations at room temperature for 60 min, before ^{51}Cr release assay. Anti-HLA-class I mAb W6/32 and anti-HLA-B,C mAb B1.23.2 were used at concentration 20 g/ml, anti-HLA-A2 mAb PA2.1 and anti-HLA-A24 mAb A11.1M were used at 1/5000 dilution of ascites. % Cytotoxicity was calculated as: % Cytotoxicity = (experimental release − spontaneous release)/(maximum release − spontaneous release) × 100. All target cells were treated with 100 IU/ml of IFN-gamma for 48–72 h before assay.

Results

Expression pro les of MHC molecules and co-stimulatory molecules of lung adenocarcinoma cell line LHK2

LHK-2, a lung adenocarcinoma cell line was established from the pleural effusion of 68-year-old male lung cancer patient. LHK2 grows stably more than 50 passages in vitro. TILs could be also obtained from the same uid using IL-2. For evaluation of its accessibility to immune system, the expression pro les of several cell surface molecules including human leukocyte antigen (HLA) and co-stimulatory molecules were evaluated with owcytometer (FCM). HLA class I molecules but not HLA class II molecules were detectable, as described previously (data not shown) (Wroblewski et al., 2001). On the other hand, one of the co-stimulatory molecules B7.1 (CD80) was not detectable with FCM (Fig. 1A). Since, lack of T cell stimulation through CD28 and B7.1 (CD80) or B7.2 (CD86) interaction can cause anergy or immunological tolerance, so, we hypothesized that these cancer cells might cause CD8$^+$ T cell anergy and survive from immune system in vivo. Thus, to evaluate the immunological meaning of B7.1 (CD80), we established B7.1 (CD80) expressing clone (LHK2-B7.1) (Fig. 1A).

CTL inducing ability of B7.1 (CD80) transfected autologous tumor cell

To establish LHK2 reactive CTLs, CD8$^+$ T cells from patient's pleural effusion were stimulated with irradiated autologous adenocarcinoma cell line, LHK2 or B7.1 introduced line (LHK2-B7.1) in the presence of IL-2 (50 IU/ml) in vitro. After 4 times of stimulation, cytotoxic activity against LHK2 and K562 as a negative control was measured with standard ^{51}Cr release assay. As shown in Fig. 1B, CD8$^+$ T cells stimulated with LHK-2 (TcLHK2 line2) showed no signi cant cytotoxic activity against LHK2, whereas CD8$^+$ T cells stimulated with LHK2-B7.1 (TcLHK2 line1) showed speci c cytotoxic activity for LHK2. This suggests that CD8$^+$ T cells from pleural effusion were anergic to LHK2 cells, and LHK2 cannot stimulate T cells anymore. However, the anergic status can be overcome by stimulation through B7.1 (CD80) on LHK2. TcLHK2 line1 recognized LHK2 speci cally in a dose dependent manner (Fig. 1C). To evaluate further the characteristics of the CTLs, inhibition assays with several mAbs were performed. As shown in

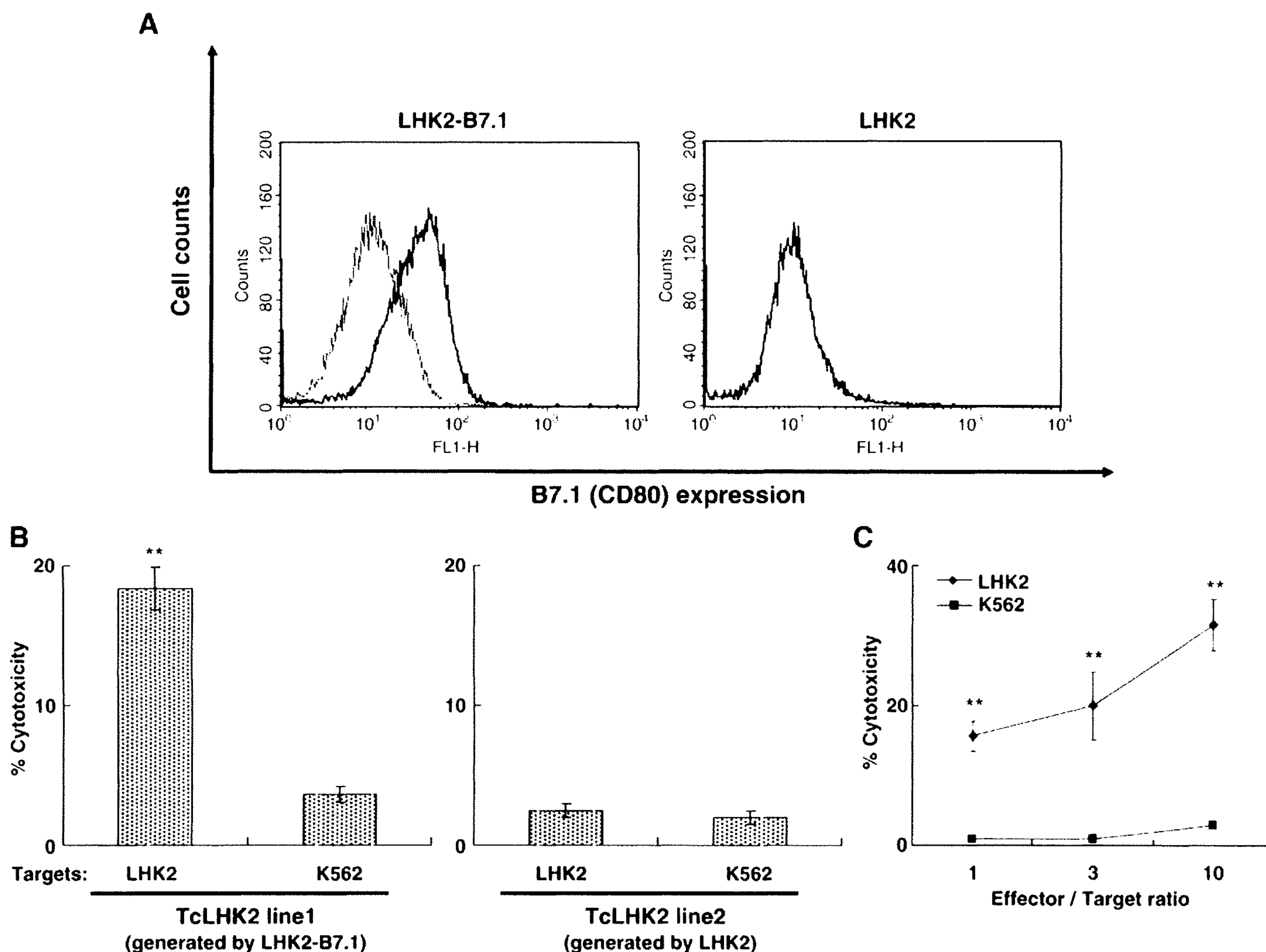

Fig. 1. (A) B7.1 (CD80) expression on LHK2 and LHK-2 B7.1 cell line. The expression of B7.1 (CD80) was evaluated by owcytometer (FCM). LHK2 and LHK2-B7.1 cell lines were labeled with anti-B7.1 antibody, following FITC-labeled 2nd antibody. The expressions were analyzed by FACSCalibur analyzer. (B) Cytotoxic activity of TcLHK2 line1 and line2. CTLs were generated by stimulating CD8$^+$ T cells using LHK2-B7.1 for TcLHK2 line1 and LHK2 for TcLHK2 line2. The cytotoxic activities for LHK2 cell line were evaluated with ^{51}Cr release assay. The effector/target ratio (E/T ratio) was 3. K562 natural killer cell target was used as a negative control. Data represent the mean ± SD. Differences between LHK2 and K562 were examined for statistical signi cance using Student's t-test. Pb0.01. (C) Cytotoxic activity. Cytotoxic activity of TcLHK2 line1 was evaluated by the ^{51}Cr release assay. The E/T ratio is 1 to 10. Data represent the mean ± SD. K562 was used as a negative control. Differences between LHK2 and K562 were examined for statistical signi cance using Student's t-test. Pb0.01.

Figs. 2A and B, the lytic activity was signi cantly inhibited by anti-HLA-class I mAb (W6/32) and anti-HLA-A24 mAb (A11.1M), but not anti-HLA-B, C mAb (B1.23.2) and anti-HLA-A2 mAb (PA2.1). These ndings indicate that TcLHK2 line1 recognizes LHK2 in an HLA-A24-restricted fashion.

Evaluation of the CTL

For further analysis of the CTL, cytotoxic activity against allogeneic tumor cells was evaluated (Table 1). TcLHK2 line1 recognized part of the allogeneic HLA-A24^{+} tumor cells including lung adenocarcinoma cell lines (PC-9), and gastric signet ring cell carcinoma cell line (KATOIII), but did not recognize other HLA-A24^{+} cells like MKN-45, SSTW, LG2-mel, LB23-SARC, OS2000, LB905-BLC and KE-4. Furthermore, cytotoxic activity against HLA-A24 transfected tumor cells was also evaluated. LNY1 is also a lung adenocarcinoma cell line, and its HLA haplotypes are completely mismatched with LHK2. Cytotoxic activity against HLA-A24 cDNA transduced LNY1 cells was signi - cantly higher than that against wild-type LNY1, on the other hand transduction of HLA-A2 and HLA-B48 did not show any difference. Same results were observed in 1353-mel. However, HLA-A24 transduced Panc-1 did not show higher sensitivity than wild-type Panc-1. These ndings suggest that TcLHK2 line1 recognized partially shared tumor antigens that were presented by HLA-A24 molecule.

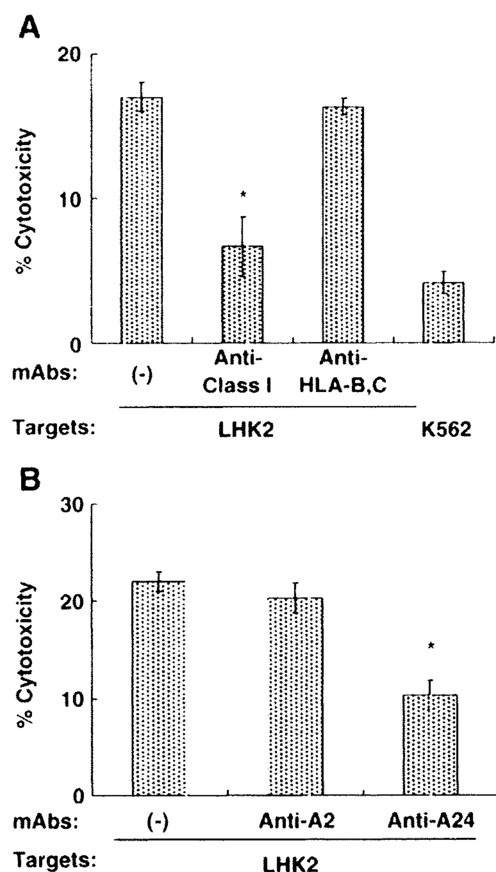

Fig. 2. HLA restriction of TcLHK2. (A) Blocking assay. The cytotoxicities were evaluated at E/T ratio = 3. Anti-HLA-class I antibody (W6/32) and anti-HLA-class I B, C locus (B1.23.2) were used for blocking assay. K562 was used as a negative control. Data represent the mean ± SD. Differences between antibody (-) and mAb groups were examined for statistical signi cance using Student's t-test. Pb0.05. (B) Blocking assay. The cytotoxicities were evaluated at E/T ratio = 3. Anti-HLA-A2 antibody (PA2.1) and anti-HLA-A24 (A11.1M) were used for blocking assay. Data represent the mean ± SD. Differences between antibody (-) and mAb were examined for statistical signi cance using Student's t-test. Pb0.05.

Table 1
Cytotoxic activity against allogeneic tumor cells.

Cell line	Histology	Origin	% CTX	HLA-A	HLA-A
LHK-2	Ad.	Lung	26.4	A 0207	A 2402
PC-9	Ad.	Lung	13.5	A 0206	A 2402
LNY-1	Ad.	Lung	2.7	A26	A31
LNY-1-A24	Ad.	Lung	11.3	A24	
LNY-1-A2	Ad.	Lung	2.7	A2	
LNY-1 B48	Ad.	Lung	3.9	B48	
MKN-45	Ad.	Stomach	2.4	A 2402	
Panc-1	Ad.	Pancreas	4.3	A 0201	A 1101
Panc-1-A24	Ad.	Pancreas	8	A24	
KATO III	Sig.	Stomach	13.6	A2	A 2402
SSTW	Sig.	Stomach	0.7	A 2402	A 2601
LG-2 MEL	Mel.	Skin	8.2	A 2402	A 3201
1353mel	Mel.	Skin	4.9	A26	A31
1353mel-A24	Mel.	Skin	13.2	A24	
LB23-SARC	Sar.	Soft tissue	7	A2	A24
OS2000	Ost.	Bone	7.4	A24	
LB905-BLC	UC.	Urinary bladder	9.3	A24	A26
KE-4	Sq.	Esophagus	2.4	A 2402	A 2601
K562	Leu		0.2		

Ad. adenocarcinoma; Sig. signet ring cell carcinoma; Mel. melanoma; Sar. sarcoma; Ost. osteosarcoma; UC. urothelial carcinoma; Sq. squamous cell carcinoma; Leu. leukemia; % CTX, % cytotoxicity; HLA, human leukocyte antigen. The % cytotoxicities were evaluated at effector/target ratio of 10. Underlines indicate the % cytotoxicities more than 10%.

Discussion

Lung cancer is one of the most frequent malignancies in the modern industrial countries. Despite recent progress in chemotherapeutic, radiotherapeutic and surgical treatment, the 5-year survival rate of lung cancer patients is still low, especially in advanced cases. Hence, new treatment modality is expected, and recent progress in understanding of tumor immunology and immunotherapy casts us the expectation that anti-lung cancer immunotherapy can be a representative treatment. For achievement of successful cancer immunotherapy, analysis of patient's immune response against lung cancer cell might be helpful.

Recently, a lot of melanoma antigens that can be recognized by CTLs have been identi ed, and several antigenic peptides had been used for immunotherapy, bringing about notable results (Rosenberg et al., 1998; Marchand et al., 1999). However, because of the dif culty of establishment of autologous tumor cell speci c CTLs, little is known about TAAs that are expressed in lung cancer cells. Hence, it is essential to analyze CTLs that recognize autologous lung cancer cell lines for establishment of new lung cancer immunotherapy.

In this study, lung adenocarcinoma cell line was established from the pleural effusion of lung cancer patient, and CD8^{+} T cells were also obtained from same pleural effusion and used for CTL induction. However, CTLs could be induced only with B7.1 (CD80) transfected LHK2 cells as an antigen-presenting cells (Fig. 1B). These results indicate that these CD8^{+} T cells were anergic against autologous tumor cells in vivo. Because CD8^{+} T cell anergy could be overcome with B7.1 (CD80) molecule, this mechanism should play major role for this tumor cells to "escape" from immune system in vivo. Takenoyama et al. (2001) also succeeded to induce CTL with B7.1 (CD80) transfected autologous lung cancer cells. Furthermore, very recently, we also succeeded to establish CTLs against autologous malignant brous hystiocytoma cell lines with B7.1 (CD80) transfected tumor cells (in preparation). Though, it is presumed that there exist several mechanisms responsible for local tolerance and irresponsibility between T cells and tumor cells, these observations might show that lacking the B7.1 (CD80) molecule is one of the major and universal mechanisms for cancer cells to escape from immune system. Importantly, these anergic statuses of CD8^{+} T cells can be reversed by B7.1 (CD80) stimulation at least in vitro (Fig. 1B). Thus, B7.1 (CD80) stimulation might be helpful for successful cancer immunotherapy.

TcLHK2 line1 recognizes autologous lung adenocarcinoma cell line in an HLA-A24-restricted fashion (Figs. 2A, B). Furthermore, this CTL can also recognize allogeneic HLA-A24^{+} adenocarcinoma, melanoma and signet ring cell carcinoma cell lines (Table 1), indicating that these CTLs recognize widely expressed shared tumor-associated antigen. Since, HLA-A24 haplotype is one of the common alleles in the world including Asian, Caucasian and African people. So, the further analysis of these CTLs-de ned antigens as well as immunological nature of these CTLs helps us to make further understanding of the mechanisms of immune-tolerance and tumor immunotherapy.

Author contributions

Y.H., T.T. and N.S. designed the research and analyzed the data. Y.H. performed most experiments. Y.H., T.T. and N.S. prepared the manuscript.

Disclosure of potential con icts of interest

All authors have declared that there are no nancial con icts of interest in regard to this work.

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Research Article

Growth Inhibition of Re-Challenge B16 Melanoma Transplant by Conjugates of Melanogenesis Substrate and Magnetite Nanoparticles as the Basis for Developing Melanoma-Targeted Chemo-Thermo-Immunotherapy

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Melanogenesis substrate, N-propionyl-cysteaminylphenol (NPrCAP), is selectively incorporated into melanoma cells and inhibits their growth by producing cytotoxic free radicals. Magnetite nanoparticles also disintegrate cancer cells and generate heat shock protein (HSP) upon exposure to an alternating magnetic field (AMF). This study tested if a chemo-thermo-immunotherapy (CTI therapy) strategy can be developed for better management of melanoma by conjugating NPrCAP on the surface of magnetite nanoparticles (NPrCAP/M). We examined the feasibility of this approach in B16 mouse melanoma and evaluated the impact of exposure temperature, frequency, and interval on the inhibition of re-challenged melanoma growth. The therapeutic protocol against the primary transplanted tumor with or without AMF exposure once a day every other day for a total of three treatments not only inhibited the growth of the primary transplant but also prevented the growth of the secondary, re-challenge transplant. The heat-generated therapeutic e ect was more significant at a temperature of 43 C than either 41 C or 46 C. NPrCAP/M with AMF exposure, instead of control magnetite alone or without AMF exposure, resulted in the most significant growth inhibition of the re-challenge tumor and increased the life span of the mice. HSP70 production was greatest at 43 C compared to that with 41 C or 46 C. CD8+T cells were infiltrated at the site of the re-challenge melanoma transplant.

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1. Introduction

Melanogenesis is inherently cytotoxic and uniquely occurs in melanocytic cells; thus, tyrosine analogs that are tyrosinase substrates are good candidates for melanoma-specific targeting and therapy [1]. *N*-propionyl and *N*-acetyl derivatives of 4-*S*-cysteaminylphenol (NPr- and NAcCAP) were synthesized and found to possess both cytostatic and cytocidal e ects on in vivo and in vitro melanomas through the oxidative stress resulting from production of cytotoxic free radicals [2–6]. We now provide evidence that the unique melanogenesis cascade can be exploited for developing a chemo-thermo-immunologic approach (CTI Therapy) targeted to melanoma by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/M).

Magnetite nanoparticles have been employed for thermotherapy in a number of cancer treatments including human prostate cancers [7–9]. They consist of 10–100 nm