Figure 2
Immunostaining of prostate cancer tissue with antibodies against AMACR and PSA. Surgically resected prostate cancer tissue was immunostained with an anti-AMACR antibody (panel A) or anti-PSA antibody (panel C). The lower column (panel B) is a magnified view of the box of panel A. A clear distinction is noted between cancerous tissue with strongly positive AMACR staining (long arrow) and noncancerous glands without AMACR staining (short arrow) whereas both of them are positive for PSA.

antigens and the immunopotent CTL epitopes. Proteins that are selectively expressed in cancer cells, but not in normal adult tissues should become suitable targets for cancer-specific immunotherapy. To establish effective immunotherapy for prostate cancer, exploration of prostate cancer-specific antigens has been conducted.

Although prostate-specific antigen (PSA) is a well-known serum biomarker for prostate cancer, it has poor specificity to cancer. PSA is highly expressed in noncancerous prostatic tissues as well as in cancerous tissues [18-20] as shown in Figure 2B in the present study. Indeed, serum PSA levels are increased in patients with benign prostatic diseases such as benign prostatic hypertrophy and prostatitis. Recently, new prostate cancer antigens have been reported and examined as target antigens for cancer-specific immunotherapy [21-23]. In the present study, we focused on AMACR, a novel antigen that is overexpressed in a variety of tumor tissues, including prostate cancer.

AMACR was identified as a tissue biomarker for prostate cancer by gene expression profiling of primary human prostate cancer and benign prostatic hyperplasia (BPH) using cDNA microarrays [8]. Initial studies reported that AMACR was overexpressed in 94-100% of prostate cancers [6-8] though recent studies have demonstrated a slightly lower expression rate in the range of 80-90% for prostate cancer [24-26]. In our study, AMACR was detected in about 70% of prostate cancer cases by immunohistochemical analysis. This frequency was slightly lower than those of previous reports. On the other hand, its expression was very low in benign prostate glands, which showed only focal and weak staining [6]. The function of AMACR in prostate cancer has not been clarified yet. It has been reported that the function and expression of AMACR might be independent of androgen receptor signaling [27]. Recently, it has been reported that AMACR is overexpressed in various tumor tissues, including renal cell cancer, hepatic cancer, colon cancer and lung cancer.
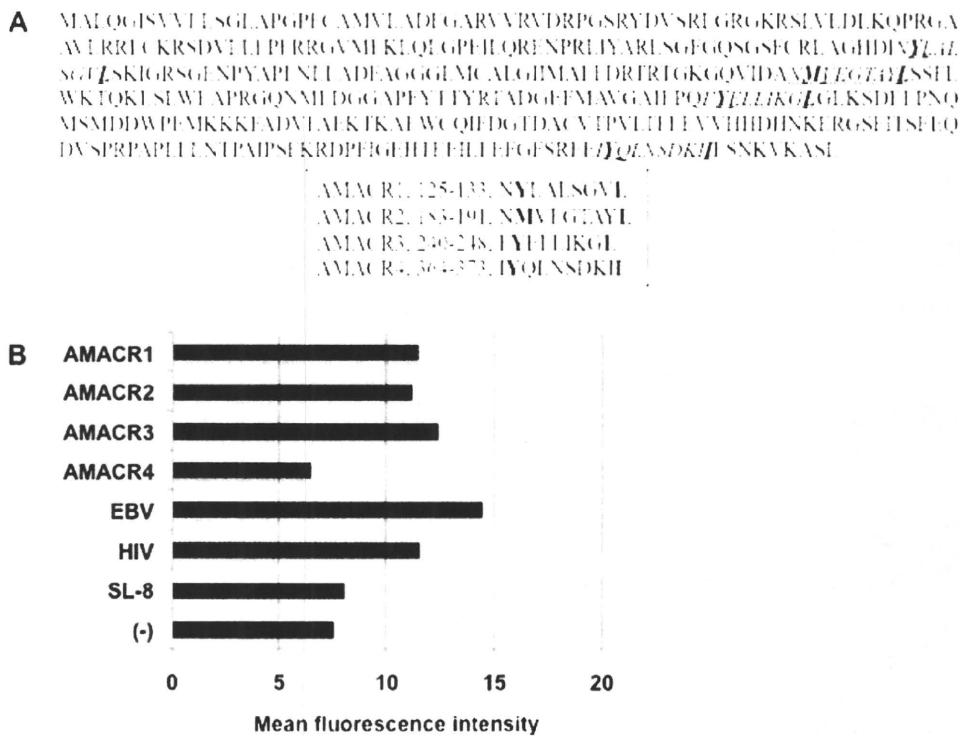
Figure 3
Amino acid sequences of AMACR-derived peptides and their HLA-A24 binding assay. A. Amino acid sequences of AMACR protein and four peptides (AMACR1-4) with HLA-A24 binding motif (underlines). The predicted anchor residues to HLA-A24 are indicated in boldface within the amino acid sequences of the peptides. B Binding affinities of AMACR-derived peptides to HLA-A24 molecule were evaluated by the mean fluorescence intensity (MFI) of cell surface HLA-A24 molecules on T2-A*2402 cells that were pulsed with each peptide. EBV LMP2-derived peptide (TYGPVFMSL) and HIV env-derived peptide (RYLRDQQLLGI) were used as positive controls for HLA-A24-bound peptides. SL-8 peptide (SIINFEKL) was used as a negative control.

Table 1: Summary of clinicopathological characteristics and peptide-specific CTL induction from the peripheral blood mononuclear cells of prostate cancer patients

Case no.	Age (years old)	PSA (ng/ml)	Gleason score	Pathologic stage	AMACR expression	CTL induction	Peptide specificity
1	60	6.7	4+3	T2aN0M0	+	+	AMACR2
2	73	6.0	3+3	T2aN0M0	+	+	AMACR2
3	65	11.6	4+3	T2bN0M0	+	+	AMACR2
4	64	15.6	3+4	T3aN0M0	+	+	AMACR1
5	67	18.4	4+5	T3aN0M0	+	+	AMACR1,2,3
6	67	14.4	4+3	T2bN0M0	+	+	AMACR1,2,3
7	71	10.9	3+5	T3bN0M0	+	-	-
8	71	4.6	3+4	T2aN0M0	+	-	-
9	72	5.7	3+4	T2aN0M0	+	-	-
10	67	8.0	4+4	T2aN0M0	+	-	-
11	67	4.3	3+3	T2bN0M0	+	-	-
12	61	11.5	3+4	T2aN0M0	-	-	-
13	61	10.1	4+3	T2bN0M0	-	-	-
14	61	10.4	3+4	T2aN0M0	-	-	-
15	60	6.6	3+4	T2aN0M0	-	-	-

▲ T2A24+AMACR1   * p<0.05
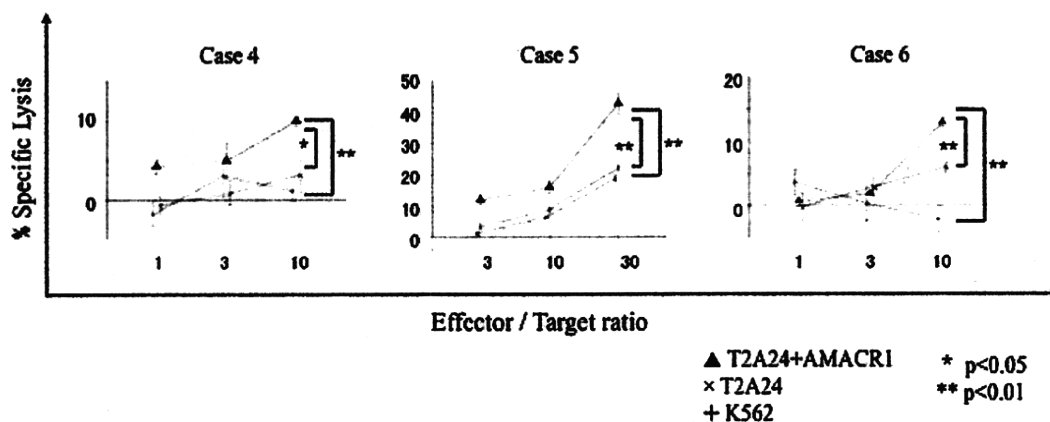× T2A24   ** p<0.01
+ K562

Figure 4
AMACR1 peptide-specific CTL induction from PBMCs of HLA-A24-positive prostate cancer patients. PBMCs of HLA-A24-positive prostate cancer patients (cases 4, 5 and 6) were stimulated four times with three kinds of AMACR peptide (AMACR1-3)-pulsed APCs and their cytotoxic activities were examined by ^{51}Cr release assay at the indicated effector/target ratios. AMACR1 peptide-pulsed T2-A*2402 cells served as target cells. Non-pulsed T2-A*2402 cells were used as negative control target cells. K562 target cells were used for monitoring natural killer cell activity and lymphokine-activated nonspecific cytotoxicity.
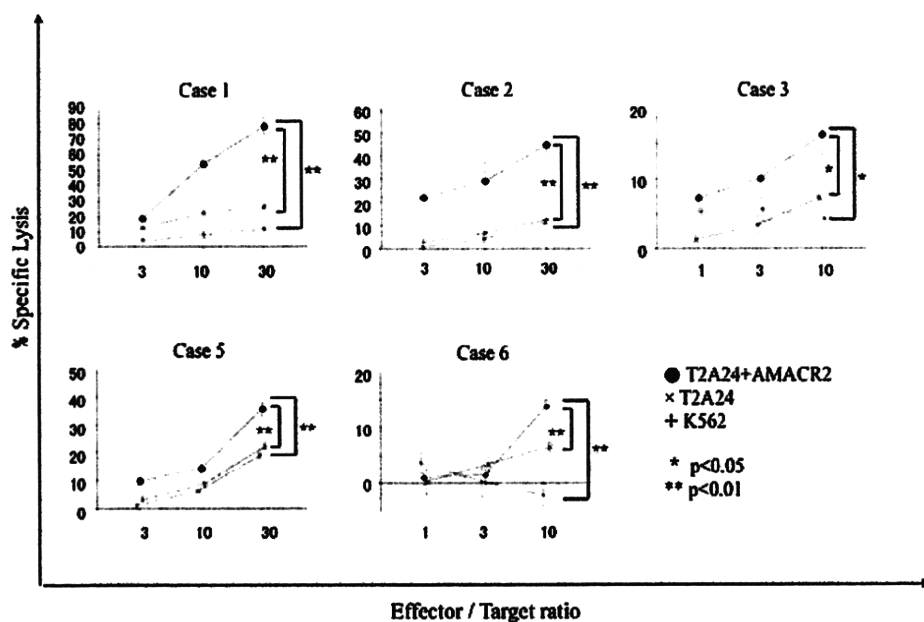

● T2A24+AMACR2
× T2A24
+ K562

* p<0.05
** p<0.01

Figure 5
AMACR2 peptide-specific CTL induction from PBMCs of HLA-A24-positive prostate cancer patients. PBMCs of HLA-A24-positive prostate cancer patients (cases 1, 2, 3, 5 and 6) were stimulated four times with three kinds of AMACR peptide (AMACR1-3)-pulsed APCs and their cytotoxic activities were examined by ^{51}Cr release assay at the indicated effector/target ratios. AMACR2 peptide-pulsed T2-A*2402 cells served as target cells. Non-pulsed T2-A*2402 cells and K562 cells were used as negative control target cells.
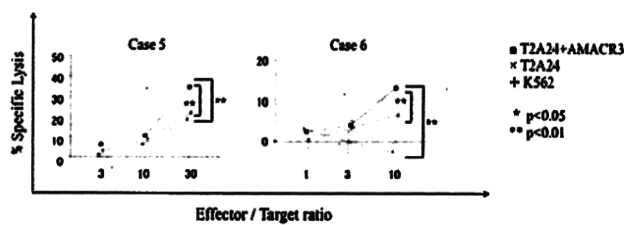
Figure 6
AMACR3 peptide-specific CTL induction from PBMCs of HLA-A24-positive prostate cancer patients. PBMCs of HLA-A24-positive prostate cancer patients (cases 5 and 6) were stimulated four times with three kinds of AMACR peptide (AMACR1-3)-pulsed APCs and their cytotoxic activities were examined by ^{51}Cr release assay at the indicated effector/target ratios. AMACR3 peptide-pulsed T2-A*2402 cells served as target cells. Non-pulsed T2-A*2402 cells and K562 cells were used as negative control target cells.

Because of the cancer specificity and high frequency of AMACR expression, it can be an attractive target for cancer immunotherapy. In this study, the immunogenic potency of AMACR-derived peptides was assessed using PBMCs from prostate cancer patients.

We focused on AMACR-derived peptides carrying the HLA-A24 binding motif. The HLA-A*2402 genotype is predominant in Japanese, accounting for about 60% of the population [28]. Four AMACR-derived peptides (AMACR1-4) carrying the HLA-A24-binding motif were identified in the present study. By stimulating peripheral blood lymphocytes of HLA-A24-positive/AMACR-expressing prostate cancer patients with these AMACR-derived peptides *in vitro*, peptide-specific CTLs were successfully induced in 4 of 9 patients. Moreover, the CTLs exerted significant cytotoxic activity against AMACR-expressing prostate cancer cells in the context of HLA-A24, indicating that AMACR-derived peptides might be useful as prostate cancer vaccines for HLA-A24-positive/AMACR-expressing prostate cancer patients. We demonstrated HLA-A24-restricted CTL responses against AMACR-derived peptides for the first time. Interestingly, the immunogenic peptides were distinct among the patients. However, it is likely that the AMACR2 peptide was the most immunogenic of the three AMACR-derived peptides.

There may be some problems in introducing new CTL-based immunotherapy for advanced recurrent and/or metastatic prostate cancer patients. Even after four rounds of *in vitro* stimulation of PBMCs with the peptides, cytotoxicity against AMACR-expressing tumor cells (% lysis) was only around 20% at a 30:1 E:T ratio. Such weak cytotoxicity may be insufficient to induce a clinical anti-tumor response. Since AMACR is involved in the bile acid syn-
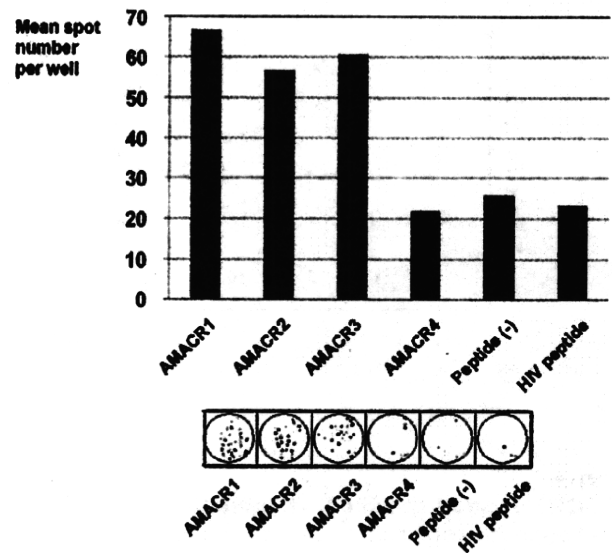

Figure 7
Peptide-specific interferon-B release of CTLs. PBMCs of HLA-A24-positive prostate cancer patient (case 6) were stimulated four times with four kinds of AMACR peptide (AMACR1-4)-pulsed APCs and peptide-specific interferon-B release was analyzed by ELISPOT assay. CTLs could release interferon-Bin response to AMACR1, 2 and 3 peptides, but not in response to AMACR4 peptide or HIV peptide.

thesis and there is weak expression in the liver, it is possible that T-cells with strong reactivity to AMACR might have tolerance to the antigenic stimulation. Thus, further studies are required to increase the cytotoxic potential of the AMACR-specific CTLs. Moreover, it is reported that AMACR expression is decreased in castration-resistant metastatic diseases [29,30]. In addition, HLA class I expression is decreased in almost 80% of prostate cancer cases as reported by us and other groups [31-33]. The down-regulation of HLA class I was observed more frequently in metastatic sites than in the primary sites. Since HLA class I has a critical role in the recognition of tumor cells by CTLs, defects in antigen presentation could allow the tumor cells to escape from killing by CTLs [34-36]. We showed previously that HLA class I down-regulation was caused at least in part by transcriptional silencing of the Γ2-microglobulin gene by histone deacetylation in prostate cancer cells, and HLA class I was restored by treatment with histone deacetylase inhibitors [33]. It may be possible for CTL-based vaccines to be used in combination with histone deacetylase inhibitors in immunotherapy for prostate cancer.

Conclusion

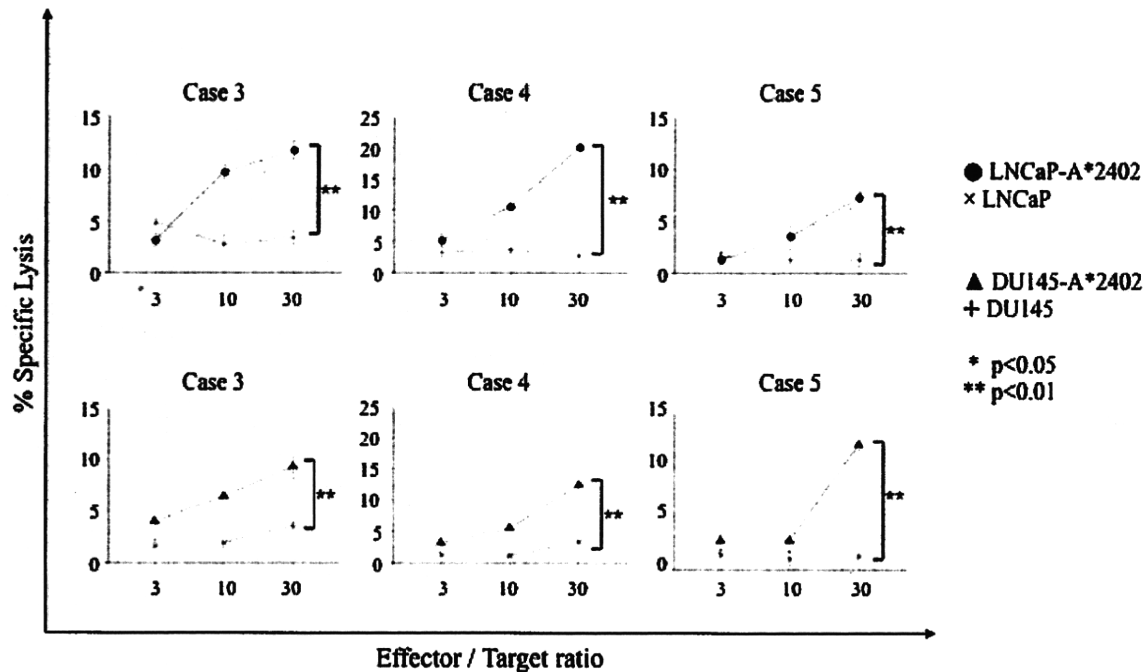In conclusion, we have provided evidence that AMACR is a potent immunogenic antigen for prostate cancer and

Figure 8
Cytotoxic activities of AMACR peptide-specific CTLs against HLA-A24-positive AMACR-expressing prostate cancer cell lines. AMACR peptide-specific CTLs were examined for the cytotoxic activity against HLA-A24-positive AMACR-expressing prostate cancer lines, LNCaP-A*2402 and DU145-A*2402, which were stable HLA-A*2402-transfectants of LNCaP and DU145, respectively. The cytotoxicity was assessed by ^{51}Cr release assay at the indicated effector/target ratios.

AMACR-derived peptides might serve as a cancer vaccine for HLA-A24-positive prostate cancer patients. It is possible that AMACR-targeting therapy might become a rational modality in immunotherapy for various AMACR-expressing cancers.

Abbreviations
AMACR: alpha-methylacyl-CoA racemase; CTL: cytotoxic T-lymphocyte; PBMC: peripheral blood mononuclear cells; DC: dendritic cell; PHA: phytohemagglutinin; APC: antigen presenting cell.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
IH carried out the CTL induction, killing assays and drafted the manuscript. TT and YH participated in the design of the study and performed the evaluation of the data. TT helped to draft the manuscript. YH contributed to the HLA-A24-binding assay and CTL induction from PBMCs. HK, ES and NM contributed to collecting patients' samples with the informed consent. YT, TT and NS contributed to the design and coordination of this study as well as reviewing the manuscript. All authors have read and approved the final manuscript.

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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 effect 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.

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 effects 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

sized iron oxide (Fe_3O_4) with a surrounding polymer coating and become magnetized when placed in an external alternating magnetic field (AMF) [10]. In hyperthermia treatment, the expression of heat shock proteins (HSPs) plays an important role in immune reactions [11–19]. Accumulating evidence from our group [20–23] and from others [24] implicates HSP expression induced by hyperthermia in tumor immunity and opens the door to cancer therapy based on hyperthermia treatment (thermo-immunotherapy). In such a strategy, a tumor-specific hyperthermia system that can induce necrotic cell death via HSP expression without damaging noncancerous tissues would be highly desirable. An intracellular hyperthermia system using tumor-targeted magnetite nanoparticles facilitates tumor-specific hyperthermia; this can induce necrotic cell death via HSP expression, which in turn induces antitumor immunity.

We synthesized, in our initial study, magnetite cationic liposomes (MCLs) loaded with 4-S-cysteaminylphenol (CAP) [25]. There was, however, a risk of nonspecific electrostatic interaction between MCLs and various non-target cells. A promising technique is the use of tumor-targeted magnetite nanoparticles, and this approach is extended by synthesizing another type of magnetite nanoparticles, NPrCAP/M, on which NPrCAP is superficially and directly bound on the surface of magnetite nanoparticles. NPrCAP/M is chemically stable and can be produced in large quantities and employed to e ect melanoma-targeted chemotherapy (by NPrCAP) and thermoimmunotherapy (by magnetite with HSP). In this study, we evaluated their thermo-therapeutic e ect on distant metastatic melanomas, using the mouse B16 melanoma system. Specifically we assessed the in vivo growth inhibition of a subsequently transplanted melanoma growth (re-challenge melanoma) after treating the initial melanoma transplant. We also investigated the possible association of HSP production with growth inhibition of the re-challenge melanoma.

2. Materials and Methods

2.1. Preparation of NPrCAP/M. The details of the preparation of NPrCAP/M are described elsewhere [26]. Briefly, magnetite nanoparticles (Fe_3O_4; average particle size, 10 nm) were coated with aminosilane and conjugated with NPrCAP via maleimide cross-linkers. The resultant NPrCAP/M was suspended in 10 mL of H_2O. The degree of incorporation of NPrCAP to magnetite was 61.0 nmol/mg magnetite.

2.2. Cells and Animal Models. All of the animal experiments were conducted by an approval of Animal Experiment Ethics Committee of Sapporo Medical University. All the surgical, transplantation and drug administration procedures were carried out after anesthesia by diethyl ether. Mouse B16F1 and B16F10 and B16 OVA melanoma cells (3.0×10^5) in 0.1 mL of phosphate-bu ered saline (PBS) were s.c. transplanted into the right flanks of 4-week-old female C57BL/6 mice (weighing approximately 10.0 g and purchased from Hokudo Laboratory, Sapporo, Japan). B16F1 and B16F10 cells were purchased from ATCC (Summit Pharmaceuticals

Intl. Corp., Tokyo, Japan). B16OVA is a B16F1 melanoma cell line stably transfected with chicken ovalbumin (OVA) cDNA and was kindly provided by Dr. Y. Nishimura, Kumamoto University, Kumamoto, Japan [27]. On day 5 after transplant mice with primary melanoma transplantation were randomly divided into treatment groups. With a 26-gauge microsyringe, the B16 melanoma-bearing mice were injected with 0.1 mL of NPrCAP/M (40.0 mg/mL solution) directly into the tumor site in a single-dose administration (approximately $0.5 \mu L/mm^3$ tumor volume). Treated tumors on the right flank in mice were excised on day 13 after the first s.c. transplantation. On day 40 after the surgical excision (on day 53 after primary transplantation), mice were re-challenged with 1.0×10^5 B16 cells which were injected into the left flank. The total number of melanoma cells at the re-challenge experiments was 1/3 of melanoma cells as that of the primary transplants because there was no NPrCAP/M administration which might cause some tissue destruction. The control group, naive mice of the same age and sex, received transplantation of melanoma cells into the right flank as with the treated groups. On day 14 after the secondary transplantation (day 67 after primary transplantation), tumor diameters were measured in millimeters with calipers, and tumor volumes were calculated by the formula: long axis × (short axis)^2 × 0.5. The mice in the treated groups were judged to be tumor free (rejection) if the tumor was less than about 2 mm in diameter by palpation on day 60 after the secondary transplantation (day 113 after the primary transplantation).

2.3. Magnetite-Nanoparticle-Mediated Hyperthermia. An alternating magnetic field (AMF) was generated using a horizontal coil (inner diameter: 7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Dai-ich High-Frequency Co., Tokyo, Japan). The magnetic field frequency and intensity were 118 kHz and 30.6 kA/m (384 Oe), respectively. Mice were exposed to the AMF inside the coil for 15 or 30 minutes. Surface (peripheral) and core (central) temperatures of the tumor were continuously monitored and measured using two optical fiber probes (FX-9020; Anritsu Meter, Tokyo, Japan); that is, one inserted into the tumor core and another fixed on the tumor surface. Measurement time points were 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 31, 32, 33, 34, and 35 minutes for 30 minutes thermotherapy. The therapeutic temperatures at 41 C, 43 C or 46 C were monitored by measuring the surface temperature and adjusting the transistor inverter during exposure to AMF (Figures 1(a), 1(b), and 1(c)).

2.4. Treatment Protocols. Animal experiments were carried out using the four protocols described below. Each experimental group of Protocols number 1 through number 4 consisted of six to eleven mice. All the treatment protocols were again approved by the Animal Experimental Ethics Committee of Sapporo Medical University. The experimental conditions for melanoma transplantation and the methods of NPrCAP/M administration were identical in all four protocols and all the animal experiments including drug
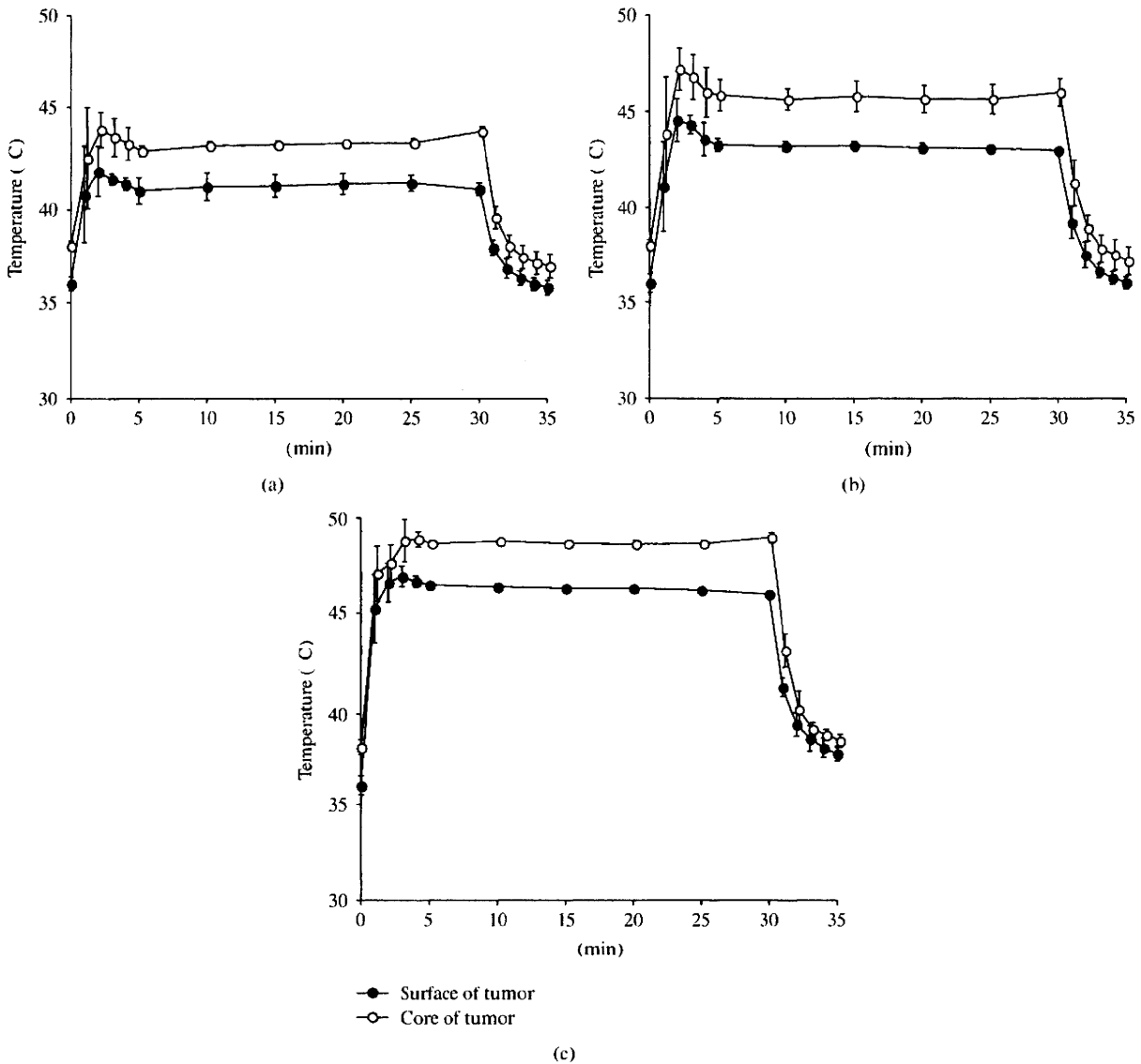
Figure 1: Shifts of temperatures at the core and the surface of tumors during AMF exposure. The temperature at the tumor surface was maintained at 41 C (a), 43 C (b), and 46 C (c) for 30 minutes by adjusting the power of the AMF generator. Temperatures of both the tumor surface and core were measured simultaneously.

administration were carried out after anesthesia by diethyl ether.

2.4.1. Protocol Number 1: E ect of NPrCAP/M with and without AMF on Re-Challenge Tumor Growth.

On day 5 after the s.c. transplantation of B16 melanoma cells, mice were divided into four groups. In Groups I and II mice received s.c. administration of 0.1 mL of 40.0 mg/mL aminosilane-coated magnetite (M) once a day every other day for a total of three days (days 6, 8, and 10) with AMF (Group II) or without AMF (Group I). In Groups III and IV mice received s.c. administration of 0.1 mL of NPrCAP/M (4.0 mg magnetite equivalent) once a day every other day for a total of three days (days 6, 8, and 10) with (Group IV) or without (Group III) AMF. The temperature at the tumor surface was maintained at 43 C during exposure for 30 minutes by controlling AMF intensity. Mice of a control group of the same age and sex received s.c. transplantation of melanoma cells into the right flank, as with the treated groups. On day 13 after the primary transplantation all mice underwent total resection of melanoma nodules. On day 53 after the first transplantation (postoperative day 40), surviving mice in each group received a second transplantation of B16
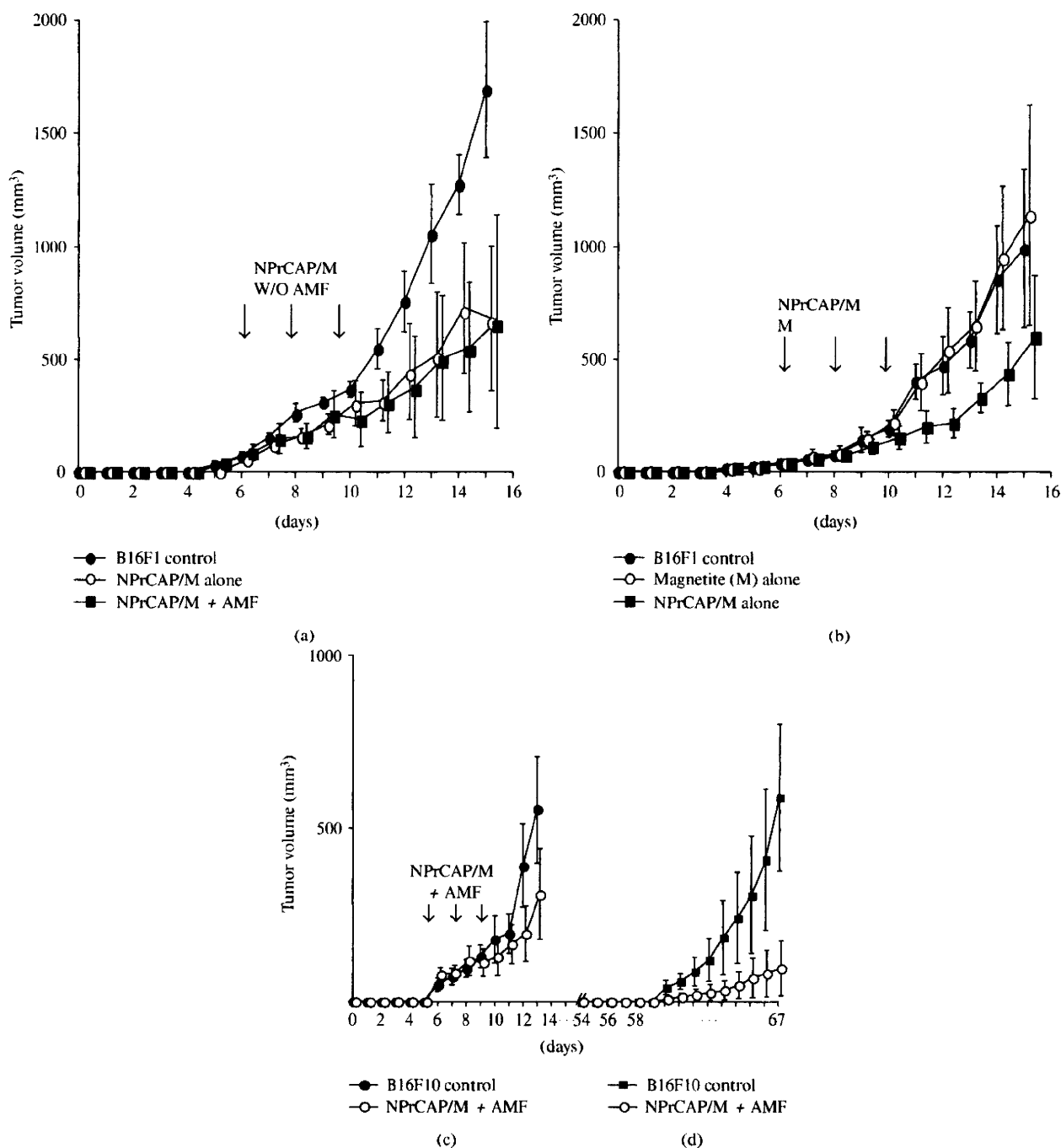
Figure 2: Growth curves of primary B16 melanomas. Experimental conditions and statistical analyses of (a), (b), (c) and (d) are identical to those of Protocol number 1. (a) Growth curve of B16F1 melanoma cells treated with NPrCAP/M alone without heat (n = 10) and with heat by AMF exposure (n = 10) at days 6, 8, and 10. These two groups showed a significant growth inhibition compared to that of control naive mice (n = 10) by Dunnet's test (P < .01). Importantly, there is no significant di erence between the two groups with and without AMF exposure. (b) Growth curve of B16F1 melanoma cells treated with magnetite alone (n = 10) and NPrCAP/M alone without AMF exposure (n = 10). Mice with magnetite alone did not show any growth inhibition whereas NPrCAP/M alone resulted in a significant growth inhibition of primary melanoma (P < .01). (c) Comparison of growth inhibition of B16F10 primary melanomas after treatment with NPrCAP/M with AMF exposure. Compared to control naive mice (n = 10) transplanted with B16F10 cells without any treatment, those mice (n = 10) with NPrCAP/M plus AMF exposure showed a significant growth inhibition of primary B16F10 melanomas with a similar degree to that of B16F1 melanoma (Figure 2(a)) (P < .01 by Dunnet's test). (d) Comparison of the growth inhibition of B16F10 secondary, re-challenge melanoma. The mice (n = 10) with NPrCAP/M plus AMF exposure showed a marked growth inhibition compared to control naive mice without any treatment (n = 10).

melanoma cells into the opposite flank. Tumor volumes were calculated at day 14 after the second transplantation of B16 melanoma cells.

2.4.2. Protocol Number 2: Effect of Treatment Frequency with NPrCAP/M on Re-Challenge Tumor Growth.
Mice were randomly divided into six treatment groups. They were exposed to AMF once on day 6 (Group I), twice (on days 6 and 8) (Group II), twice (on days 6 and 10) (Group III), three times (on days 6, 8, and 10) (Group IV), three times (on days 6, 7, and 8) (Group V), and five times (on days 6 through 10) (Group VI).

2.4.3. Protocol Number 3: Effect of Temperature and Treatment Frequency of NPrCAP/M with AMF on Re-Challenge Tumor Growth.
Mice were divided into six groups. Mice of Groups I and II were exposed to the AMF to maintain the surface temperature at 41 C once a day for two days (days 6 and 10) and for three days (days 6, 8, and 10), respectively. Using the same day schedule mice of Groups III and IV were exposed to the AMF at 43 C and mice of Groups V and VI at 46 C.

2.4.4. Protocol Number 4: Effect of Temperature and Treatment Duration on Re-Challenge Tumor Growth.
Mice were divided into four groups. Temperatures at the surface of the tumors in Groups I and II were maintained at 43 C and 46 C, respectively, during AMF exposure for 15 minutes. The surface temperatures in Groups III and IV were maintained at 43 C and 46 C, respectively, during therapy for 30 minutes.

2.5. ELISA for Heat Shock Protein 70 (HSP70) Expression in a Tumor.
After thermotherapy, the amount of HSP70 in the primary tumor was measured using an HSP70 EIA Kit (Stress Gen Biotechnologies, British Columbia, Canada) according to the manufacturer's instructions. The total protein content of the tumor homogenates was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Inc., USA). The control group was composed of mice without NPrCAP/M administration or AMF exposure. Group I received s.c. administration of NPrCAP/M directly at the tumor site once a day without AMF exposure. Mice of Groups II and III received thermotherapy at 41 C for 15 minutes and 30 minutes, Groups IV and V at 43 C for 15 minutes and 30 minutes, and Groups VI and VII at 46 C for 15 minutes and 30 minutes, respectively. Then, 24 hours later, all tumors were removed, and their lysates were processed for the HSP70 assay. In separate groups, tumors were excised at 24, 48, and 72 hours after the 43 C thermotherapy for 30 minutes, and amounts of HSP70 were measured.

2.6. Histological and Immunohistochemical Study.
After thermotherapy in the primarily transplanted melanoma at 43 C for 30 minutes once a day for three days (Figure 4(a), Group IV), melanomas in re-challenge mice were excised on the 18th day after second transplantation of B16F1 cells. Paraffin-embedded sections were prepared and processed for HE staining. The frozen tumor tissues were stained with antimouse CD4 (Santa Cruz Biotechnology Inc., CA, USA) or CD8 (Chemicon International Inc., CA, USA).

2.7. Statistical Analysis.
Data were analyzed by one- or two-way analysis of variance (ANOVA), and then differences in experimental results for tumor growth and expression of HSP 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. Differences in survival rates were analyzed by the Kaplan-Meier method and log-rank test with Bonferroni correction for multiple comparisons. The level of significance was $P < .05$ (two-tailed). All statistical analyses were performed using Stat View J-5.0 (SAS Institute Inc. Cary, NC).

3. Results and Discussion

In the search for successful cancer treatment it is self-evident that the exploitation of a specific biological property is one of the best approaches for developing the targeted therapy [27, 28]. We have previously shown that the melanogenesis substrate, NPrCAP, is a good candidate for developing melanoma chemotherapy because melanogenesis is uniquely expressed in melanocytic cells and is inherently cytotoxic from the action of tyrosinase on tyrosine with formation of highly reactive free radicals [1, 4]. Nanoparticles may also provide a good platform to coadminister anticancer therapeutics directed at different targets. Specifically hyperthermia with the use of magnetite nanoparticles has been shown to possess great potential to develop thermo-immunotherapy [23, 29]. In this study the conjugate of NPrCAP and magnetite nanoparticles, NPrCAP/M was synthesized with the hope of developing a chemotherapeutic as well as a thermo-immunotherapeutic effect. We employed two cell lines of B16 melanoma, that is, B16F1 and B16F10, and B16OVA cells and compared the thermo-therapeutic protocols in detail by evaluating the growth of the re-challenge melanoma as well as the duration and rates of survival of melanoma-bearing mice.

3.1. Immediate and Steady Generation of Heat by Intratumor Administration of NPrCAP/M with AMF Exposure on B16 Melanoma Nodules.
In the previous intratumor MCL hyperthermia for B16 melanoma the skin surface temperature above the subcutaneous tumor rose to 43 or 46 C [29]. To obtain a rapid and steady temperature increase at the core and the surface of the B16 melanoma, NPrCAP/M was injected into the center of the tumor nodules, and internal and surface tumor temperatures were measured during AMF exposure. Both temperatures increased within one minute to the target of 41 C, 43 C, or 46 C (Figures 1(a), 1(b), and 1(c)), indicating that NPrCAP/M injection followed by AMF exposure could immediately and steadily heat the subcutaneously transplanted melanoma nodules. The temperature at the tumor center was approximately 2 C higher than that at the tumor surface.
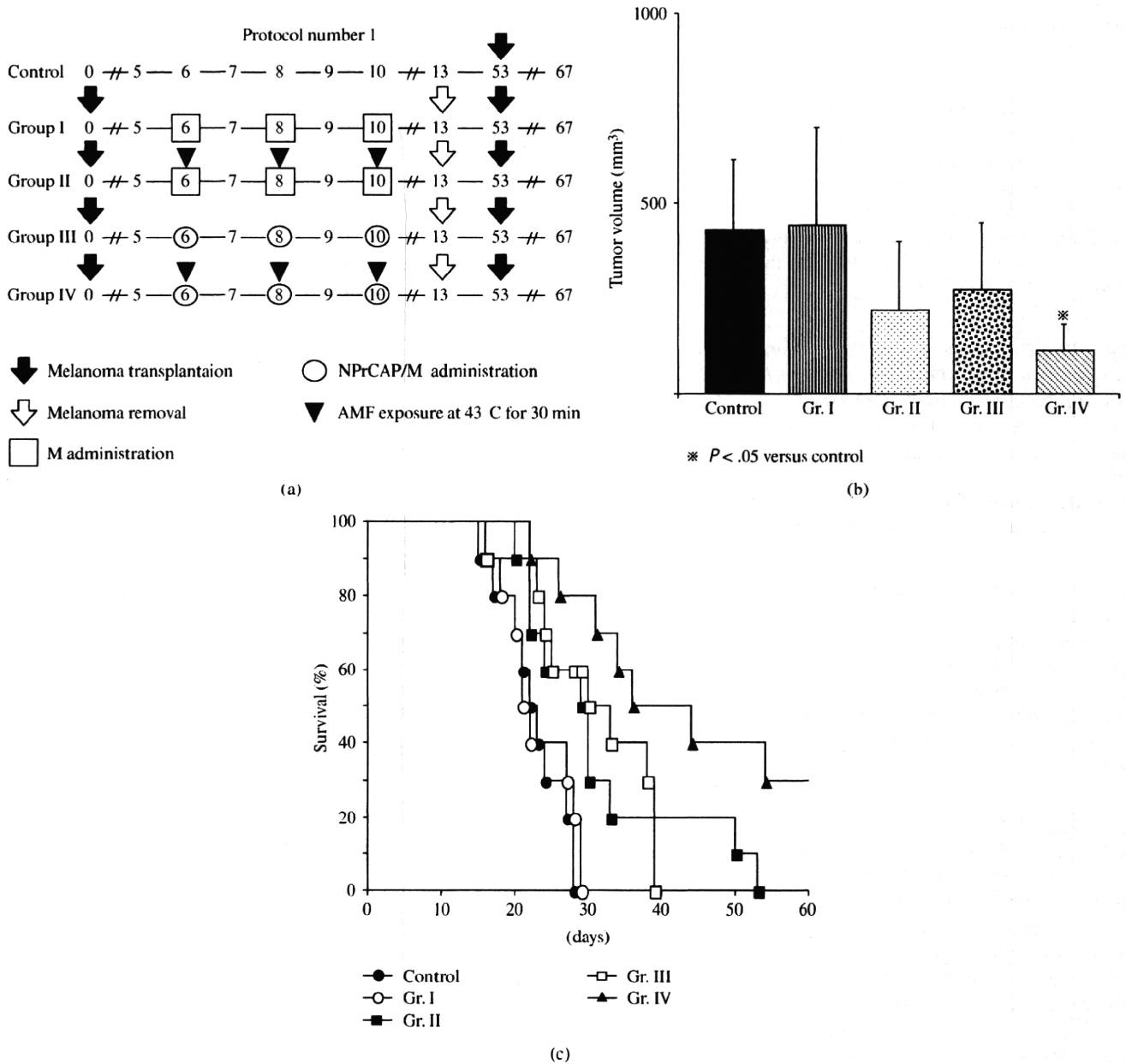
(a)

(b)

(c)

Figure 3: Time schedules and results for tumor volumes, survival periods, and rates of mice treated by the Protocol number 1. (a) Protocols of Groups I, II, III, and IV. (b) Tumor volumes of re-challenge B16F1 melanoma on day 14. All data are presented as mean ± standard deviation. Tumor volumes of Group IV were significantly reduced compared with those of the control group (P = .0295) or Group I (P = .0215). There were no significant interactions between drugs and AMF (P = .5568). (c) Kaplan-Meier survival curve over a period of 60 days after tumor re-challenge in Protocol number 1.

3.2. E ective and Equal Inhibition of B16 Melanoma Growth at the Site of Primary Transplantation by Intratumor Administration of NPrCAP/M with and without Heat.

We first evaluated the chemotherapeutic e ect of NPrCAP/M with or without heat. NPrCAP/M without heat inhibited the growth of primary transplants to the same degree as did NPrCAP/M with heat, indicating that NPrCAP/M alone has a chemotherapeutic e ect. The critical temperature for thermotherapy was documented to be 43 C for various cell lines [7, 8]. Using two melanoma cell lines, B16F1, and B16F10 and B16OVA, we examined melanoma growth inhibition by intra-tumor administration of NPrCAP/M into primary tumors on days 6, 8, and 10 after transplantation with exposure to AMF at 43 C for 30 minutes (Figures 2(a) and 2(c)) under the experimental conditions of Protocol number 1 (Figure 3(a)). Both NPrCAP/M with and without AMF exposure resulted in a significant and equal reduction of melanoma tumor volume in both B16F1 and F10 cells
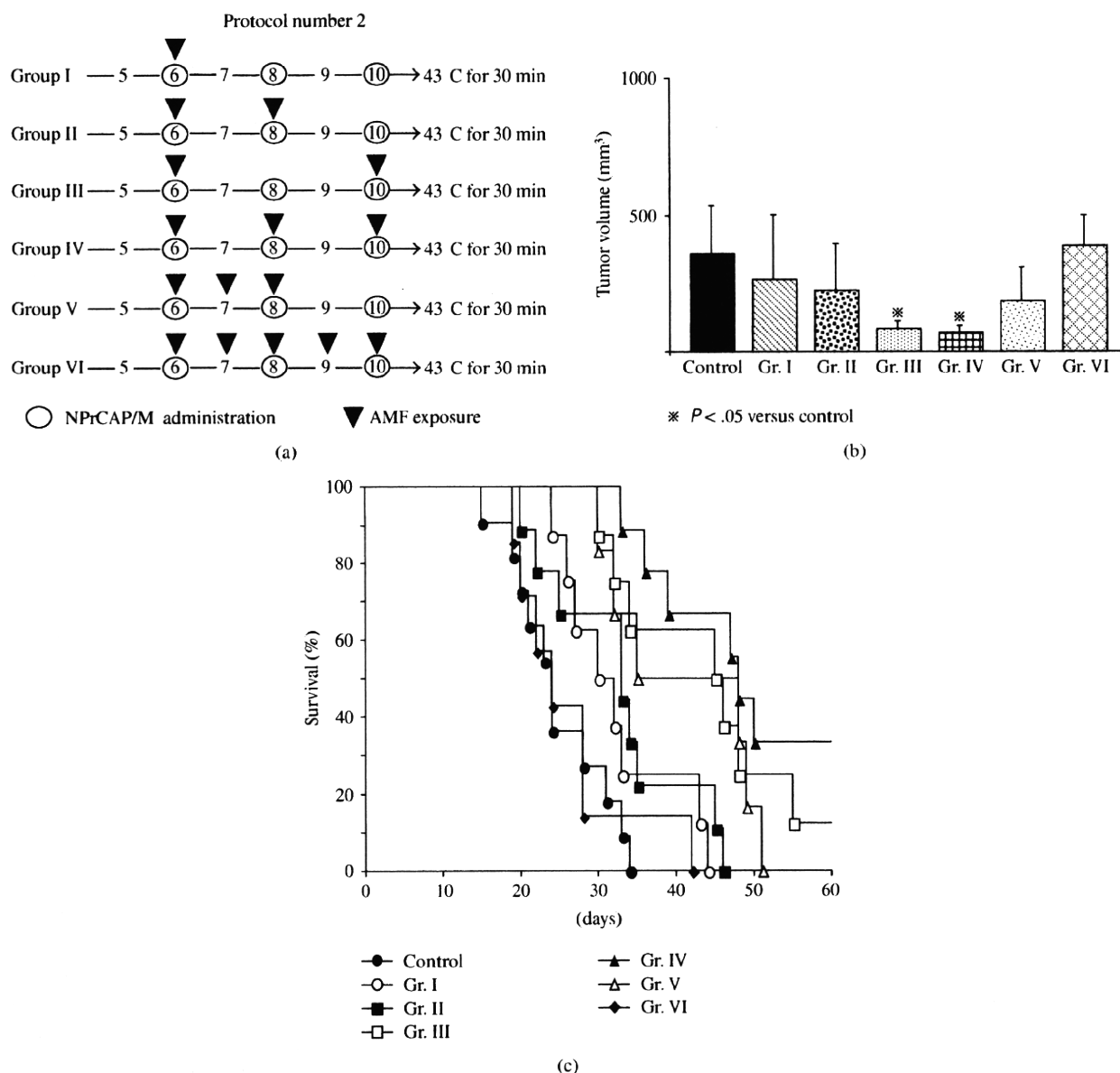
Figure 4: Time schedule and results for tumor volumes, survival periods, and rates of mice treated by Protocol number 2. (a) Protocols of Groups I, II, III, IV, V, and VI. (b) Tumor volumes on day 14 after re-challenge with B16F1 melanoma. All data are presented as mean ± standard deviation. Tumor volumes of Groups III and IV were found to be significantly reduced compared with those of the control group (P = .0411 and .0195, resp.) and Group VI (P = .0444 and .0237, resp.) by the Scheffé test. (c) Kaplan-Meier survival curves over a period of 60 days after re-challenge with B16F1. The survival rate of Group III was significantly prolonged compared with those of the control group (P = .0006) and Group VI (P = .0013). The survival rate of Group IV was significantly prolonged compared with that of the control group (P < .0001), Group I (P = .0014), Group II (P = .0014), and Group VI (P = .0013). One of the eight mice in Group III and three of the nine mice in Group IV were protected against re-challenge with B16F1 melanoma cells.

at the site of primary transplantation (P < .01 by two-way repeated measure ANOVA, Figures 2(a) and 2(c)) compared to tumor volume of naive control mice. B16 OVA cells also gave the same experimental results (data not shown). Control studies comparing magnetite alone and magnetite plus NPrCAP (NPrCAP/M) without AMF

exposure showed that magnetite alone does not have any melanoma growth inhibiting effect whereas NPrCAP/M without AMF significantly inhibited melanoma-growth (P < .01 by two-way repeated measure ANOVA, Figure 2(b)). Since we obtained basically same growth inhibition results for both the primary and secondary transplants from the
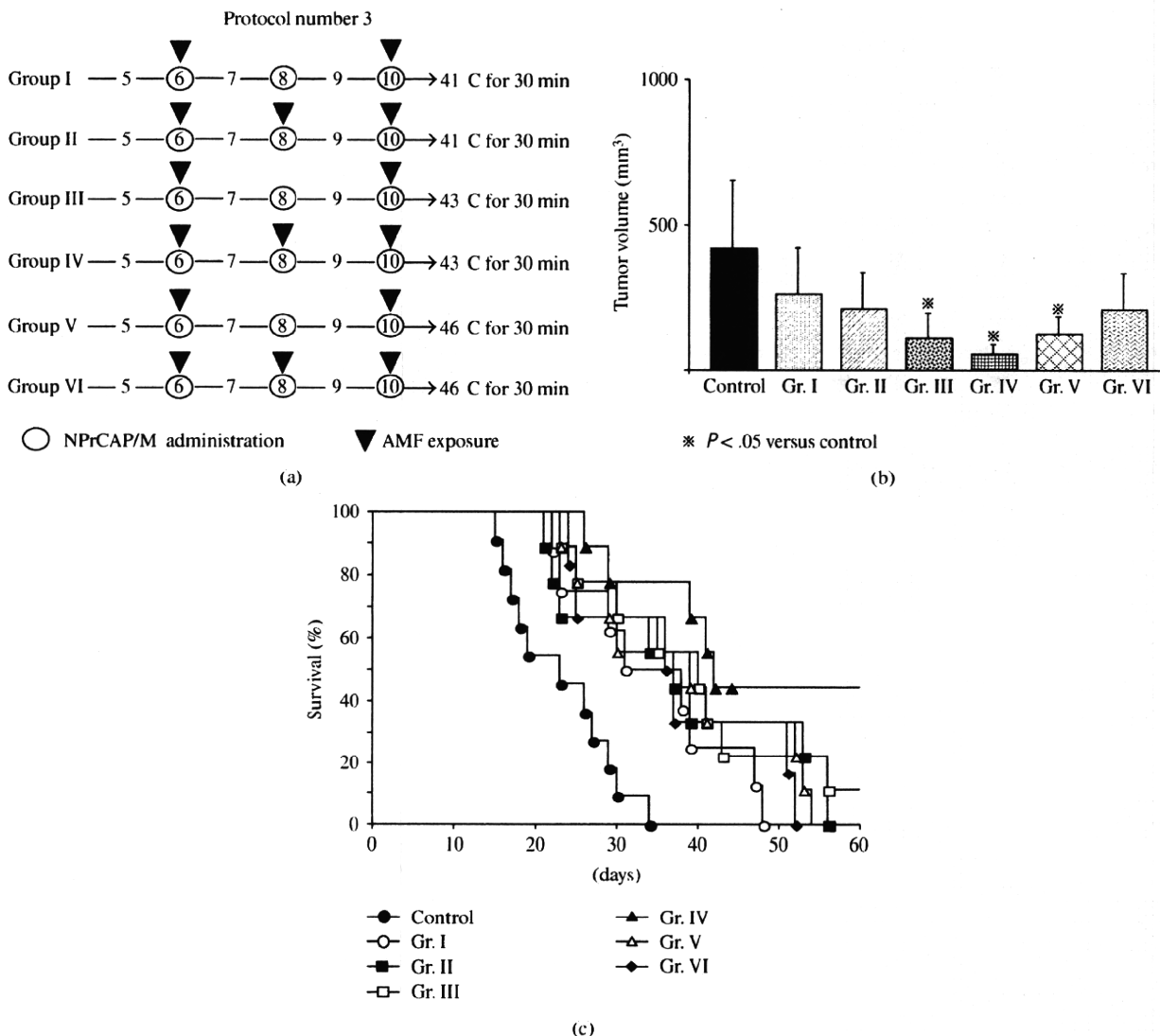
(a)

(b)

(c)

Figure 5: Time schedules and results for tumor volume, survival periods and rates of mice treated by Protocol number 3. (a) Protocols of Groups I, II, III, IV, V, and VI. (b) Tumor volumes on day 14 after re-challenge with B16F1 melanoma. All data are presented as mean ± standard deviation. Tumor volumes of Groups III, IV, and V were significantly reduced compared with the control group (P = .0045, .0004, and .0085 resp.) by the Sche é test. (c) Kaplan-Meier survival curve over a period of 60 days after tumor re-challenge. Survival rates of Groups III and IV were significantly prolonged compared with that of the control group (P = .0011 and .0002, resp.). One of the nine mice in Group III and four of the nine mice in Group IV were protected against re-challenge with B16F1 melanoma cells.

two cell lines, the majority of subsequent studies listed in Protocols number 1 through number 4 were conducted on B16F1 cells.

3.3. E ective Growth Inhibition of B16F1 Melanoma Cells at the Site of Re-Challenge, Second Transplantation by NPrCAP/M with AMF Exposure (Protocol number 1). We then evaluated whether NPrCAP/M treatment with or without heat in the local primary tumor could inhibit the growth of distant tumors which were not given an intra-tumor injection of NPrCAP/M. There was a significant

di erence in the melanoma growth inhibition of re-challenge transplants between the groups of NPrCAP/M with and without heat. NPrCAP/M with AMF exposure showed the most significant growth inhibition in re-challenge melanoma and increased life span of the host animals, that is, 30%–50% complete growth inhibition (rejection) of re-challenge melanoma growth, indicating that NPrCAP/M with heat possesses a thermo-immunotherapeutic e ect. For this, we treated the primary B16F1 and F10 melanoma cells by NPrCAP/M and then measured the volume of the secondary melanoma after the second transplantation at a di erent site to the first transplant. We also evaluated the survival periods
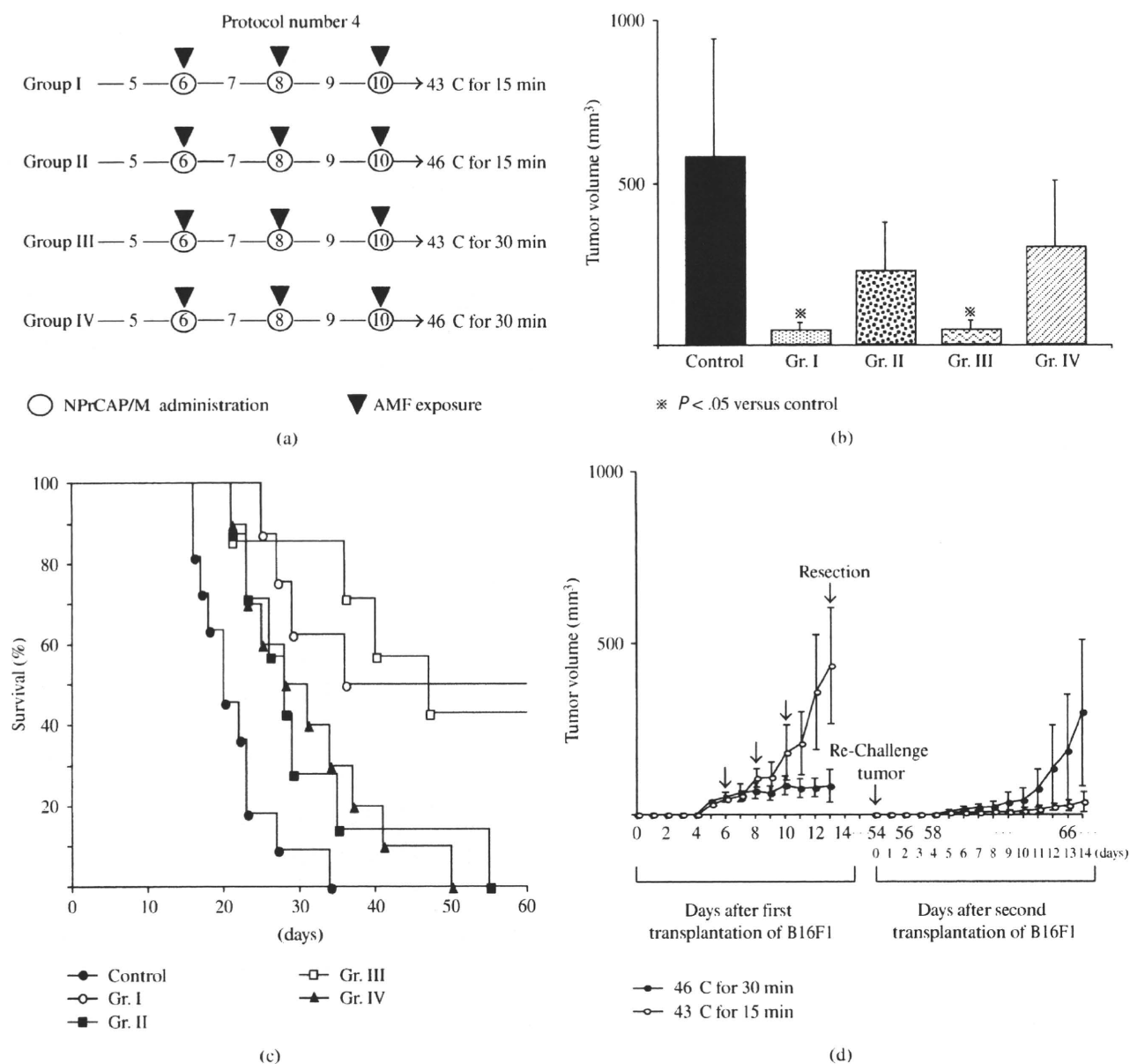
Figure 6: Time schedules and results for tumor volumes, survival periods, and rates for treatment with Protocol number 4. (a) Protocols of Groups I, II, III, and IV. (b) Tumor volumes on day 14 after re-challenge with B16F1 cells. All data are presented as mean ± standard deviation. Tumor volumes of Groups I and III on day 14 were significantly reduced compared with that of the control group (P = .0009 and .0016, resp.) by Scheffé test. (c) Kaplan-Meier survival curve over a period of 60 days after re-challenge. Survival rates of Group I and III were significantly prolonged compared with that of the control group (P = .0004 and .0005, resp.). Four of the eight mice in Group I and three of the seven mice in Group III were protected against re-challenge with B16F1 melanoma cells. (d) Tumor volumes of the primary tumor and re-challenge tumor as representative examples of Groups I (n = 8) and IV (n = 10) which were treated at 43 C for 15 minutes and 46 C for 30 minutes, respectively. All data are presented as mean ± standard deviation.

and rates of host melanoma-bearing mice. These secondary melanomas were not directly exposed to NPrCAP/M; hence we could evaluate the thermo-immunotherapeutic effect of NPrCAP/M treatment.

First, we compared the therapeutic effects among Groups I (intratumor injection of magnetite nanoparticles alone

without AMF exposure), II (magnetite injection and heat at 43 C for 30 minutes by AMF), III (NPrCAP/M injection without AMF), and IV (NPrCAP/M injection and heat at 43 C for 30 minutes by AMF) of Protocol number 1 (Figure 3(a)). As shown in Figure 3(b), NPrCAP/M-mediated hyperthermia at 43 C showed the most significant
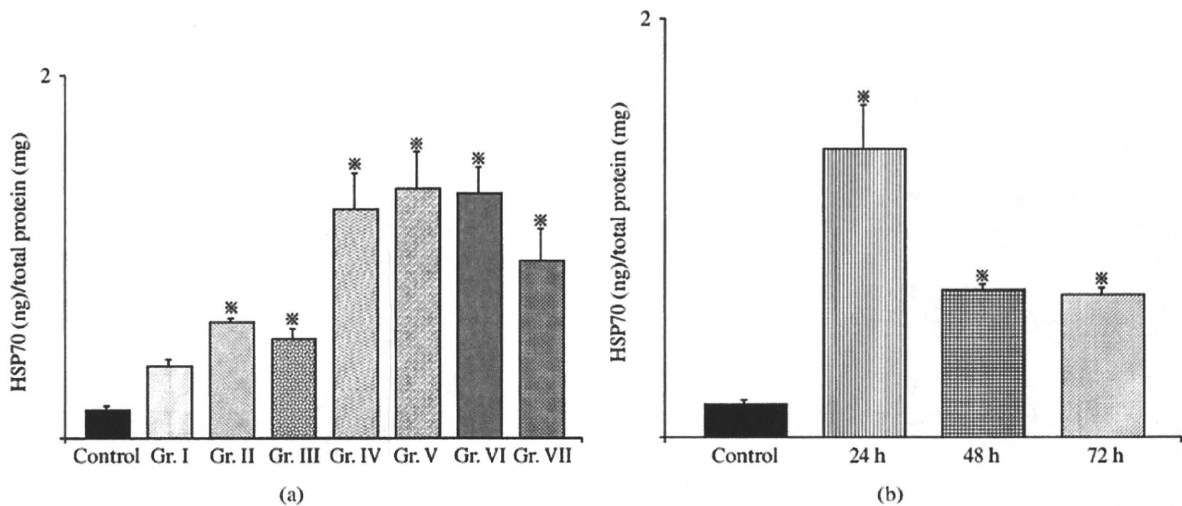
(a)

(b)

Figure 7: Expression of HSP70 in a tumor after thermotherapy. (a) Amounts of HSP70 in tumors 24 hours after thermotherapy as described in the Materials and Methods. All data are presented as mean ± standard deviation (n = 4). There were significant differences between the control group and all other groups except Group I by Dunnett's test (P < .05). (b) Amounts of HSP70 24, 48, and 72 hours after thermotherapy at 43 C for 30 minutes. All data are presented as mean ± standard deviation (n = 4). There were significant differences between the control group and all other groups by Dunnett's test (P < .05).
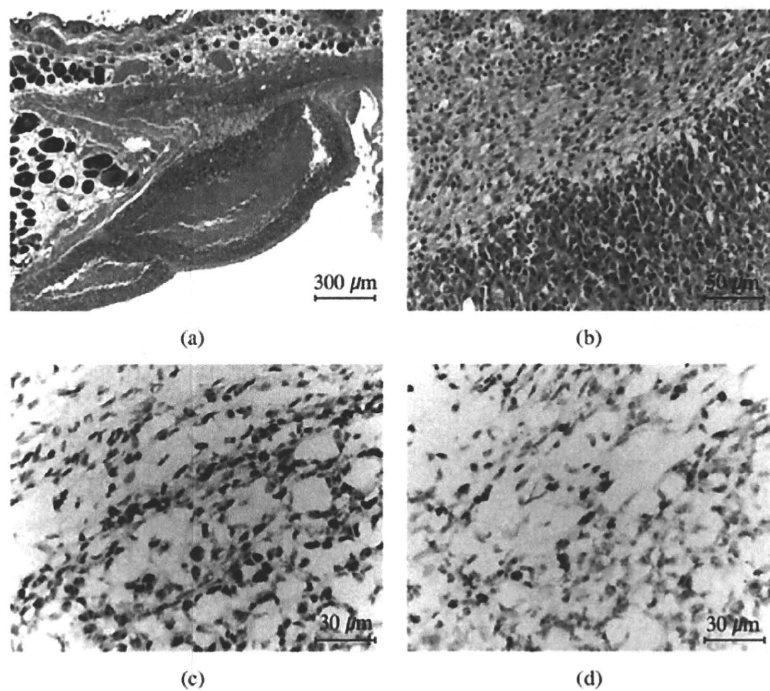

(a)

(b)

(c)

(d)

Figure 8: Histopathology and immunohistochemistry of a re-challenge tumor. (a) A low-power view of a re-challenge tumor with HE staining (×40). (b) A high-power view (×200). Monocytic infiltrates are seen around a necrotic lesion. (c) CD4+ T cells (×400). (d) CD8+T cells (×400). Almost equal numbers of CD4+ and CD8+ T cells are observed.

growth inhibition of secondary B16F1 melanoma in re-challenged mice. Both magnetite nanoparticles with heat at 43 C and NPrCAP/M without heat also inhibited the growth of secondary melanomas, though statistically not significant (Figure 3(b) and (c)). Most importantly, NPrCAP/M alone without heat caused equal growth inhibition of secondary melanomas to that induced by magnetite with AMF exposure, suggesting some immunotherapeutic effect of NPrCAP/M. A similar growth inhibition of secondary transplanted melanoma cells was obtained in B16F10 (Figure 2).

Next, we compared the life span of the host animals among 4 groups. The survival of mice in Group IV was prolonged, compared with that of the control group (P = .0003) and Group I (P = .0003). Three of the ten mice in Group IV (30%) were protected completely from re-challenge with B16F1 cells (Figure 3(c)). Magnetite alone with AMF exposure at 43 C (Group II) and NPrCAP/M alone without heat (Group III) failed to show any statistically significant prolongation of the host animal survival.

3.4. E ect of Treatment Frequency for the Primary Tumor on Growth Inhibition of Re-Challenge Melanoma (Protocol number 2).

To evaluate the e ect of the number of treatments for the primary tumor on the re-challenge tumor, six treatment approaches were designed using B16F1 cells. They consisted of hyperthermia once on day 6 (Group I), twice on days 6 and 8 (Group II) or days 6 and 10 (Group III), three times on days 6, 8, and 10 (Group IV) or 6, 7, and 8 (Group V), and a total of five times on days 6 through 10 (Group VI) (Figure 4(a)). Melanoma tumor volumes in re-challenged mice were smallest in Groups III and IV, while the longest survival periods and rates were obtained in Group IV with complete growth inhibition (rejection) of second re-challenge being 33% (n = 9) on day 60 (Figures 4(b) and 4(c)). The consecutive irradiation on days 6, 7, and 8 (Group V) or days 6 through 10 resulted in larger volumes of secondary tumors and poorer survival periods and rates compared to Group III or IV (Figures 4(b) and 4(c)). These findings suggested that repeated hyperthermia, once a day every other day for a total of three days, could induce e ective degradation of B16 melanoma cells, which then most likely induced host immunity against melanoma.

3.5. E ect of Temperature and Treatment Frequency on Melanoma Growth Inhibition in Re-Challenge Mice (Protocol number 3).

Our study indicated that the most e ective thermo-immunotherapy for re-challenge B16 melanoma can be obtained at a temperature of 43 C for 30 minutes with the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma. We compared growth inhibition of secondly transplanted melanomas at therapeutic temperatures of 41 C, 43 C, and 46 C for 30 minutes twice on days 6 and 10, or three times on days 6, 8, and 10 (Figure 5(a)). As shown in Figures 5(b) and 5(c), thermotherapy at 43 C in Group IV (43 C, every other day for a total of three times on three days) was the most e ective for the growth inhibition of both the secondly transplanted, re-challenge melanoma and for improving the survival rates and duration of host mice. Four of the nine mice in Group IV (44.4%) were protected completely against re-challenge with B16F1 melanoma on day 60 (Figure 5(c)).

Our therapeutic conditions and their e ects di er from those of magnetically mediated hyperthermia on the transplanted melanomas reported previously [29]. MCL-mediated hyperthermia for B16 melanoma showed that hyperthermia at 46 C once or twice led to regression of 40%–90% of primary tumors and to 30%–60% survival

of mice, whereas hyperthermia at 43 C failed to induce regression of the secondary tumors with 0% survival of mice [29].

3.6. E ect of Temperature and Treatment Duration on Melanoma Growth Inhibition in Re-Challenge Mice (Protocol number 4).

We then compared the e ects of temperature and duration of NPrCAP/M-mediated hyperthermia at 43 C for 15 minutes, 43 C for 30 minutes, 46 C for 15 minutes, and 46 C for 30 minutes on the re-challenge with B16F1 melanoma (Figure 6(a)). Tumor volumes and survival rates and periods of treatment of mice clearly showed that hyperthermia at 43 C elicited a more significant e ect than that at 46 C (Figures 6(b) and 6(c)). Four of the eight mice (50%) in Group I (43 C for 15 minutes) and three of the seven mice (42.8%) in Group III (43 C for 30 minutes) survived 60 days after a second transplantation of B16F1 (Figure 6(c)), suggesting that NPrCAP/M with heat to the primary melanoma at 43 C for 15–30 minutes inhibits significantly the growth of distant metastatic melanoma, complete growth inhibition (rejection) of the second re-challenge melanoma being 42%–50%. Hyperthermia at 46 C for 30 minutes strongly inhibited the growth of the B16F1 tumor but had little e ect on the re-challenge tumor, whereas hyperthermia at 43 C for 15 minutes hardly inhibited the growth of the primary tumor but strongly inhibited that of the second re-challenge tumor (Figure 6(d)). These findings suggest that NPrCAP/M-mediated hyperthermia at 43 C can be used most e ectively to treat distant metastatic melanoma.

3.7. Production of HSP70 by NPrCAP/M Treatment and Presence of CD8+ T Cells around and within the Re-Challenge Melanoma.

Heat shock protein forms a complex with intracellular peptides released from degrading tumor cells and presented by the MHC class I molecules of professional antigen-presenting cells [23]. We analyzed HSP70 production in the primary tumor and CD4+ and CD8+ T cell infiltration into the re-challenge secondary tumor. Figure 7(a) shows the amounts of HSP70 in the tumors at 24 hours after the NPrCAP/M-mediated hyperthermia. Among the six treatment groups, conditions of hyperthermia at 43 C for 15 or 30 minutes and 46 C for 15 minutes were equally e ective for induction of HSP70 as those at 41 C for 15 minutes or 30 minutes and at 46 C for 30 minutes (Figure 7(a)). We also investigated whether expression of HSP70 in the posttherapeutic tumors depended on the duration of AMF exposure (15 minutes or 30 minutes), heating temperature (41 C, 43 C, or 46 C), and time elapsed after exposure (24 hours, 48 hours, or 72 hours). Figure 7(b) shows that the amount of HSP70 in the treated B16F1 tumors was most abundant at 24 hours after hyperthermia at 43 C, and over-expression of HSP70 was maintained at a significant level after 72 hours. Although thermotherapy at 46 C for 15 minutes could induce HSP70 as abundantly as that at 43 C for 30 minutes (Figure 7(a)), this condition failed to suppress the re-challenge melanoma transplant as

e ciently as 43 C thermotherapy (Figures 5(b) and 5(c)). This suggests that immunological factors other than HSPs are at least in part responsible for growth inhibition and rejection of the re-challenge melanoma. Hyperthermia at 43 C for 1 hour mediated the expression of MHC class I molecules after 24 hours in association with enhanced expression of HSP70 [30]. Heat treatment of tumor cells permits enhanced cross-priming, possibly via up-regulation of both HSPs and tumor antigen expression [24]. By inducing HSP70 and possibly MHC class I, immune T cells could aggregate around melanoma cells. We thus examined histochemically the immunological reaction against secondly transplanted, re-challenge B16F1 melanoma in hematoxylin and eosin (HE)- and CD4- and CD8-stained sections. In addition to neutrophilic leukocytes, macrophages, and plasma cells, $CD4^+$ and $CD8^+$ T cells were observed around and within the re-challenge tumors with necrotic lesions (Figures 8(a), 8(b), 8(c), and 8(d)). These T cells were seen with a small number around the first transplant melanoma treated by NPrCAP/M with or without AMF exposure but hardly observed around the naive B16F1 tumors in mice that were not treated by NPrCAP/M-mediated thermotherapy (data not shown). This may indicate that melanoma-specific T cell immunity is likely involved in our NPrCAP/M therapy strategy.

4. Conclusions

This study has provided the basis for developing a melanoma targeted chemo-immuno-thermotherapy (CTI) strategy by conjugating melanogenesis substrate, NPrCAP with magnetite nanoparticles after exposure to alternating magnetic field. NPrCAP/M-mediated hyperthermia at a relatively low temperature (43 C) e ectively inhibited the growth of second transplant, re-challenge melanoma. Possibly, superficially bound NPrCAP possesses important roles in targeting nanoparticles to melanocytic cells and a chemotherapeutic e ect on these cells. Based upon the present animal therapeutic protocol, that is, three-every-other-day treatment at 43 C, we have started preliminary clinical trials (phase I/II) of NPrCAP/M CTI therapy with a significant success to a limited number of advanced stages III and IV melanoma patients. Four patients entered in this trial after approval of the Ethics Committee of Sapporo Medical University and two of them showed PR and CR, still surviving and carrying out normal daily life for more than 24 months [31].

Lastly, it should be noted that melanin intermediates produce reactive oxygen species such as superoxide and H_2O_2 [4, 32, 33]. This unique biological property of melanin intermediates not only causes cell death but also may produce immunogenic properties. In fact, NPrCAP/M alone without heat was as e ective as magnetite nanoparticles with AMF exposure in inhibiting growth of re-challenge melanoma (Figures 3(b) and 3(c)). It would be interesting to know whether the growth of secondary re-challenge melanoma could be inhibited after treatment of NPrCAP alone onto the primary tumor [34]. The molecular background of our NPrCAP/M CTI therapy needs to be further studied.

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Experimental and Molecular Pathology

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

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article info

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