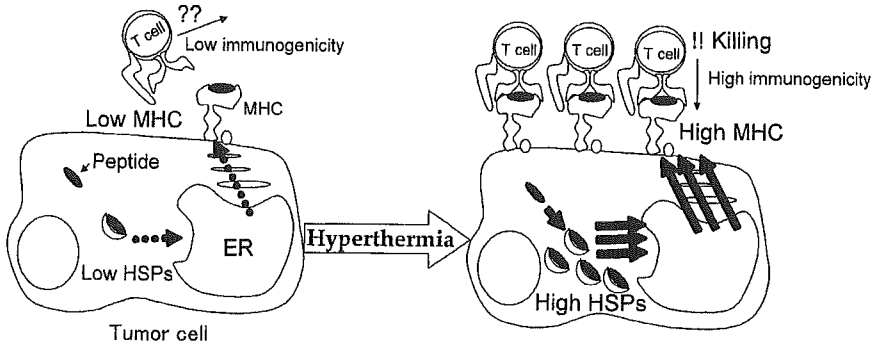

Fig. 3. Augmentation of tumor immunogenicity by an increase of the number of MHC molecules on the surface of cancer cells via inducible HSP expression during hyperthermia.

Vaccine-like effect of HSP-peptide complex released from necrotic cells during hyperthermia

An alternative mechanism of recognition of tumor antigens by the host immune system in hyperthermia is cross-presentation of antigenic peptides by dedicated antigen-presenting cells (APCs), including dendritic cells (DCs). The working hypothesis is illustrated in Fig.4. HSP-mediated antitumor immunity may be caused by a vaccine-like effect of HSP-peptide complexes released from dying tumor cells. The released HSP-peptide complexes encounter APCs that express receptors such as CD91 [32], CD40 [33] and Toll-like receptors 2/4 [34]. Interaction of HSP-peptide complexes with these receptors leads to receptor-mediated endocytosis, processing of the antigenic peptide by the endogenous MHC class I pathway, and re-presentation on the cell surface to CD8-positive T cell receptors. Additionally, HSP70 functions as a direct activator of APCs, stimulating cytokine secretion from monocytes and inducing the maturation of DCs via CD14 and/or CD91 receptors [35]. This cytokine-like ability of HSP70 to stimulate the innate immune system is independent of the peptides they chaperone, suggesting that HSP70 is a natural adjuvant [36) 37].

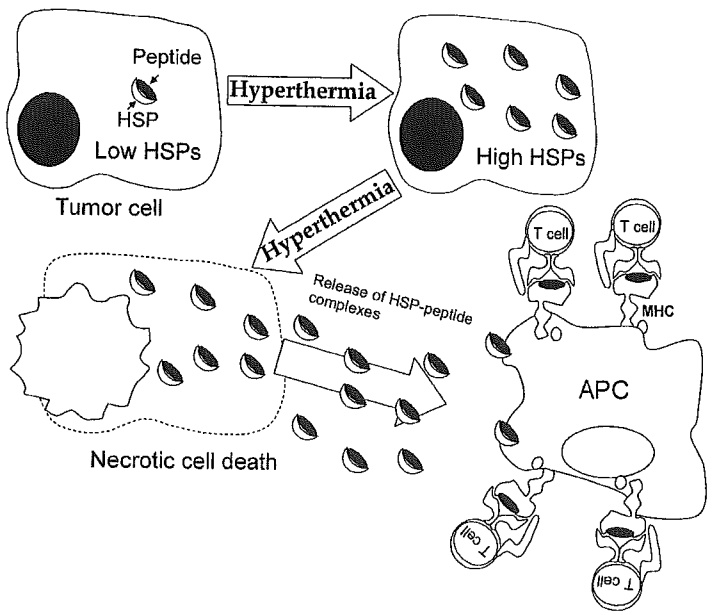

Fig. 4. Mechanism of anti-cancer immune induction by hyperthermia. In situ vaccination by HSP-peptide complexes released from dying cells via necrosis by hyperthermia treatment

Classical immunology theory is based on the distinction between "self and nonself". However, this self-nonself model is sometimes not very useful, particularly in cancer immunity because cancer

cell is derived from normal cell that is "self" originally. Matzinger proposed the "Danger model", which suggests that the immune system is more concerned with damage than with foreignness [38]. In the "Danger model", HSPs are considered an important "Danger signal". In particular, when HSPs, which are stress-indicators and normally present in cytoplasm, are released into the bloodstream, they can act as a signal to the immune system of an abnormal situation. We demonstrated that HSP70 expression following hyperthermia using MCLs induced antitumor immunity in rats with T-9 rat glioma [18]. Because MCLs are themselves heated in our hyperthermia system, the distribution of magnetite nanoparticles within tumors is an important issue. When MCLs were repeatedly heated, the surrounding tumor tissues underwent necrosis, and magnetite nanoparticles subsequently expanded into the necrosis area within the tumor, resulting in wide distribution of magnetite nanoparticles. Thus, the entire tumor area was necrosed by repeated (3 times) hyperthermia (with a 24-h interval over 3 days) [2]. The 24-h interval corresponded to the time when HSP70 expression in T-9 cells reached its maximum, and a large amount of HSP70 was detected in the tumor tissue. Thus, our hyperthermia system using MCLs overcame thermotolerance and induced necrotic cell death that correlated with HSP70 expression. A reason why necrotic cell death via HSP70 expression is effective for cancer therapy is that necrotic cell death may strongly induce a "Danger signal". We observed that numerous and diverse kinds of immunocytes, such as CD8- and CD4-positive T-cells, NK cells, macrophages and DCs, infiltrated into the necrotic area of tumors after hyperthermia treatment [2) 18]. Todryk et al observed infiltration of such cells into B16 melanoma nodules transfected with the HSP70 gene [39], suggesting that HSP70 expression is a "Danger signal" for recruitment of immunocytes. Next, we purified the HSP70-peptide complexes from the tumor after hyperthermia, and found that immunization of rats with T-9 HSP70 strongly suppressed tumor growth [2]. HSP70-peptide complexes from liver were also purified, and their vaccine-like effects were examined, but no anti-tumor effects were observed. These results suggest that HSP70 in tumor cells chaperons some antigenic peptides after hyperthermia. Also, in order to investigate the vaccine-like effect of the tumor cells killed via hyperthermia-induced necrosis in rats, a tumor rejection assay was performed after hyperthermia treatment of implanted T-9 cells; tumor growth was strongly suppressed, and 50% of the rats were protected from challenge with T-9 cells.

Recently, a vaccine consisting of autologous tumor-derived gp96-peptide complexes (HSPPC-96, Oncophage; Antigenics, Inc, Woburn, MA) has entered clinical trials, and the feasibility of its use to treat metastatic melanoma patients has been demonstrated [40]. Because HSP-peptide complexes must be extracted from tumors in the body, surgery is needed in this therapeutic protocol. In contrast, in hyperthermia using MCLs, no surgery or extraction is necessary. Udono *et al.* reported that the vaccination effect of HSP70-peptide complexes was directly dependent on the dose [41]. The HSPs in the tumor can be regarded as an antigen source, and 1 g (approximately 10^8 cells) of tumor tissue may contain approximately 2 mg HSP70. This represents a much higher dose than that used for the clinical trials. In our hyperthermia system, the expression of HSP70 was enhanced, and tissue lysis via necrosis was observed throughout the tumor. Our hyperthermia system using MCLs can produce HSP-peptide complexes (possibly including HSPs such as HSP90 and gp 96), resulting in vaccination.

These results suggest that our hyperthermia system confers antitumor immunity via release of HSP70-peptide complexes during necrotic tumor cell death *in vivo*. This phenomenon, which may be

called *in situ* vaccination, has important implications for development of novel anti-tumor therapies.

Conclusion

A proposed scenario in which HSPs function during successive stages of an antitumor response after hyperthermia is summarized and illustrated in Fig.5 : 1) A poorly immunogenic tumor cell with a low concentration of intracellular HSP-peptide complexes, decreased function of the endogenous antigen processing machinery, and a very low level of MHC class I-peptide complexes at the cell surface. 2) A sublethal stress response induced by hyperthermia using MCLs results in increased levels of intracellular HSP-peptide complexes, enhanced processing of endogenous antigens, and an increase in the density of MHC class I-peptide complexes at the cell surface. These tumor cells are then recognized directly by MHC class I-restricted CTLs. 3) Dying tumor cells, which are killed by the CTLs or by lethal hyperthermia treatment, release their intracellular contents, including HSP-peptide complexes. 4) The released HSPs and/or antigenic peptides activate neighboring monocytes to produce proinflammatory cytokines and recruit APCs. 5) The HSP-peptide complexes are taken up by DCs, and are in turn presented to T cells via MHC class I and/or II antigens (cross-priming).

The mechanism of antitumor immune response induced by hyperthermia was studied by using T-9 rat glioma cells that are known to show relatively high immunogenicity. Currently, we use oncogene *RET*-transgenic mice [42] in order to investigate whether the antitumor immunity can be induced in the mice in which melanoma spontaneously develops, as a hereditary melanoma model of low immunogenicity. Several melanoma nodules spontaneously developed in a RET-transgenic mouse in 3 month after birth. Interestingly, although only one tumor was subjected to hyperthermia, some of the

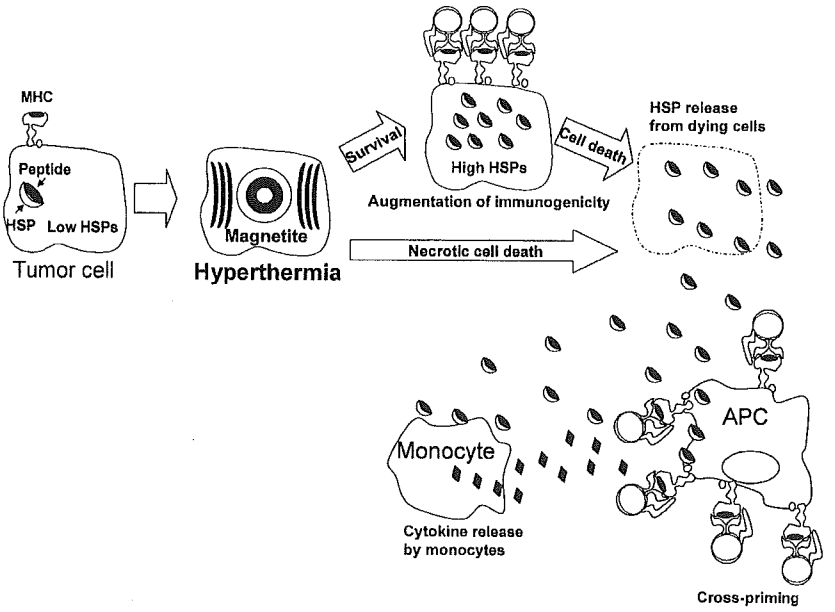

Fig. 5. Proposed scenario of the mechanism of anti-cancer immune induction by hyperthermia.

other tumors (3/7 tumors in a mouse) also disappeared completely (unpublished results). Further investigations to elucidate the mechanisms are ongoing.

We are currently developing novel cancer immunotherapy based on the mechanism of anti-cancer immune response via HSP expression. Three key elements may be involved in this mechanism : 1) CTL as an effector cell, 2) APC as an antigen-processing and -presenting cell for HSP-peptide complex released from necrotic cells, 3) HSPs as a natural and powerful immunostimulant. This strategy is based on combinations of hyperthermia using MCLs with cytokines (IL-2 and GM-CSF [43]), heat-inducible TNF-α gene therapy [44], recombinant HSP70 [45], HSP70 gene therapy [46], and DC therapy (unpublished results). We are conducting further studies to establish a novel cancer immunotherapy based on the concept of heat-controlled necrosis with HSP expression.

Acknowledgements This work was partly supported by Grants-in-Aid for Scientific Research (No. 13853005), University Start-Ups Creation Support System, and the 21st Century COE Program "Nature-Guided Materials Processing" from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1) Ito A., Shinkai M., Honda H., Wakabayashi T., Yoshida J., Kobayashi T. : Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia. Cancer Immunol Immunother, 50 : 515-522, 2001.
2) Ito A., Shinkai M., Honda H., Yoshikawa K., Saga S., Wakabayashi T., Yoshida J., Kobayashi T. : Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles. Cancer Immunol Immunother, 52 : 80-88, 2003.
3) Cavaliere R., Ciocatto E. C., Giovanella B. C., Heidelburger C., Jonson R. O., Margottini M., Mondovi B., Moricca B. G., Rossi-Fanelli A. : Selective heat sensitivity of cancer cells. Biochemical and clinical studies. Cancer, 20 : 1351-1381, 1967.
4) Ikeda N., Hayashida O., Kameda H., Ito H., Matsuda T. : Experimental study on thermal damage to dog normal brain. Int J Hyperthermia, 10 : 553-561, 1994.
5) Stauffer P. R., Cetas T. C., Fletcher A. M., DeYoung D. W., Dewhirst M. W., Oleson J. R., Roemer R. B. : Observations on the use of ferromagnetic implants for inducing hyperthermia. IEEE Trans Biomed Eng, 31 : 76-90, 1984.
6) Jordan A., Wust P., Fahling H., John W., Hinz A., Felix R. : Inductive heating of ferrimagnetic particles and magnetic fluids : physical evaluation of their potential for hyperthermia. Int J Hyperthermia, 9 : 51-68,1993.
7) Moroz P., Jones S. K., Gray B. N. : Magnetically mediated hyperthermia : current status and future directions. Int J Hyperthermia, 18 : 267-284, 2002.
8) Gordon R. T., Hines J. R., Gordon D. : Intracellular hyperthermia. A biophysical approach to cancer treatment via intracellular temperature and biophysical alterations. Med Hypothesis, 5 : 83-102, 1979.
9) Le B., Shinkai M., Kitade T., Honda H., Yoshida J., Wakabayashi T., Kobayashi T. : Preparation of tumor-specific magnetoliposomes and their application for hyperthermia. J Chem Eng Jpn, 34 : 66-72, 2001.
10) Shinkai M., Le B., Honda H., Yoshikawa K., Shimizu K., Saga S., Wakabayashi T., Yoshida J., Kobayashi T. : Targeting hyperthermia for renal cell carcinoma using human MN antigen-specific magnetoliposomes. Jpn J Cancer Res, 92 : 1138-1145, 2001.
11) Ito A., Kuga Y., Honda H., Kikkawa H., Horiuchi A., Watanabe Y., Kobayashi T. : Magnetite nanoparticle-loaded

anti-HER2 immunoliposomes for combination of antibody therapy with hyperthermia. Cancer Lett, 212 : 167-175, 2004.

12) Shinkai M., Yanase M., Honda H., Wakabayashi T., Yoshida J., Kobayashi T. : Intracellular hyperthermia for cancer using magnetite cationic liposome : in vitro study. Jpn J Cancer Res, 87 : 1179-1183, 1996.

13) Suzuki M., Shinkai M., Honda H., Kobayashi T. : Anticancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes. Melanoma Res, 13 : 129-135, 2003.

14) Ito A., Tanaka K., Honda H., Abe S., Yamaguchi H., Kobayashi T. : Complete regression of mouse mammary carcinoma with a size greater than 15 mm by frequent repeated hyperthermia using magnetite nanoparticles. J Biosci Bioeng, 96 : 364-369, 2003.

15) Yanase M., Shinkai M., Honda H., Wakabayashi T., Yoshida J., Kobayashi T. : Intracellular hyperthermia for cancer using magnetite cationic liposome : an in vivo study. Jpn J Cancer Res, 89 : 463-469,1998.

16) Matsuoka F., Shinkai M., Honda H., Kubo T., Sugita T., Kobayashi T. : Hyperthermia using magnetite cationic liposomes for hamster osteosarcoma. Biomagn Res Technol, 2 : 3, 2004.

17) Matsuno H., Tohnai I., Mitsudo K., Hayashi Y., Ito M., Shinkai M., Kobayashi T., Yoshida J., Ueda M. : Interstitial hyperthermia using magnetite cationic liposomes inhibit to tumor growth of VX-7 transplanted tumor in rabbit tongue. Jpn J Hyperthermic Oncol, 17 : 141-149, 2001.

18) Yanase M., Shinkai M., Honda H., Wakabayashi T., Yoshida J., Kobayashi T. : Antitumor immunity induction by intracellular hyperthermia using magnetite cationic liposomes. Jpn J Cancer Res, 89 : 775-782, 1998.

19) Lindquist S. : The heat-shock response. Ann Rev Biochem, 55 : 1151-1191, 1986.

20) Mosser D. D., Caron A. W., Bourget L., Meriin A. B., Sherman M. Y., Morimoto R. I., Massie B. : The chaperon function of hsp 70 is required for protection against stress-induced apoptosis. Mol Cell Biol, 20 : 7146-7159, 2000.

21) Subjeck J. R., Sciandra J. J., Chao C. F., Johnson R. J. : Heat shock proteins and biological response to hyperthermia. Br J Cancer, 45 : 127-131, 1982.

22) Subjeck J. R., Sciandra J. J., Johnson R. J. : Heat shock proteins and thermotolerance ; a comparison of induction kinetics. Br J Radiol, 55 : 579-584, 1982.

23) Menoret A., Chandawarkar R. : Heat-shock protein-based anticancer immunotherapy : An idea whose time has come. Sem Immunol, 25 : 654-660, 1998.

24) Srivastava P. K., Menoret A., Basu S., Binder R., Quade K. : Heat shock proteins come of age : primitive functions acquired new roles in an adaptive world. Immunity, 8 : 657-665, 1998.

25) Tamura Y., Sato N. : Heat shock proteins : chaperoning of innate and adaptive immunities. Jpn J Hyperthermic Oncol, 19 : 131-139, 2003.

26) Srivastava P. K., Maki R. G. : Stress-induced proteins in immune response to cancer. Curr Top Microbiol Immunol, 167 : 109-123, 1991.

27) Srivastava P. K., Heike M. : Tumor-specific immunogenicity of stress-induced proteins : convergence of two evolutionary pathways of antigen presentation? Semin Immunol, 3 : 57-64, 1991.

28) Srivastava P. K., Udono H., Blachere N. E., Li Z. : Heat shock proteins transfer peptides during antigen processing and CTL priming. Immunogenetics, 39 : 93-98, 1994.

29) Wells A. D., Rai S. K., Salvato M. S., Band H., Malkovsky M. : Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. Int Immunol, 10 : 609-617, 1998.

30) Wells A. D., Malkovsky M. : Heat shock proteins, tumor immunogenicity and antigen presentation : an integrated view. Immunol Today, 21 : 129-132, 2000.

31) Okamoto M., Tazawa K., Kawagoshi T., Maeda M., Honda T., Sakamoto T., Tsukada K. : The combined effect against colon-26 cells of heat treatment and immunization with heat treated colon-26 tumour cell extract. Int J Hyperthermia, 16 : 263-273, 2000.

32) Basu S., Binder R. J., Ramalingam T., Srivastava P. K. : CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity, 14 : 303-313, 2001.

33) Becker T., Hartl F. U., Wieland F. : CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. J Cell Biol, 158 : 1277-1285, 2002.

34) Asea A., Rehli M., Kabingu E., Boch J. A., Bare O., Auron P. E., Stevenson M. A., Calderwood S. K. : Novel signal transduction pathway utilized by extracellular HSP70 : role of toll-like receptor (TLR) 2 and TLR4. J Biol Chem, 277 : 15028-15034, 2002.

35) Asea A., Kraeft S. K., Kurt-Jones E. A., Stevenson M. A., Chen L. B., Finberg R. W., Koo G. C., Calderwood S. K. : HSP70 stimulates cytokine production through a CD14-dependent pathway, demonstrating its dual role as a chaperon and cytokine. Nat Med, 6 : 435-442, 2000.

36) Srivastava P. K. : Roles of heat-shock proteins in innate and adaptive immunity. Nat Rev Immunol, 2 : 185-194, 2002.

37) Srivastava P. K. : Interaction of heat shock proteins with peptides and antigen presenting cells : chaperoning of the innate and adaptive immune responses. Annu Rev Immunol, 20 : 395-425, 2002.

38) Matzinger P. : The danger model : a renewed sense of self. Science, 296 : 301-305, 2002.

39) Todryk S., Melcher A. A., Hardwick N., Linardakis E., Bateman A., Colombo M. P., Stoppacciaro A., Vile R. G. : Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. J Immunol, 163 : 1398-1408, 1999.

40) Belli F., Testori A., Rivoltini L., Maio M., Andreola G., Sertoli M. R., Gallino G., Piris A., Cattelan A., Lazzari I., Carrabba M., Scita G., Santantonio C., Pilla L., Tragni G., Lombardo C., Arienti F., Marchiano A., Queirolo P., Bertolini F., Cova A., Lamaj E., Ascani L., Camerini R., Corsi M., Cascinelli N., Lewis J. J., Srivastava P., Parmiani G. : Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes : clinical and immunologic findings. J Clin Oncol, 20 : 4169-4180, 2002.

41) Udono H., Srivastava P. K. : Comparison of tumor-specific immunogenicities of stress-induced protein gp96, hsp90, and hsp70. J Immunol, 152 : 5398-5403, 1994.

42) Kato M., Takahashi M., Akhand A. A., Liu W., Dai Y., Shimizu S., Iwamoto T., Suzuki H., Nakashima I. : Transgenic mouse model for skin malignant melanoma. Oncogene, 17, 1885-1888, 1998.

43) Ito A., Tanaka K., Kondo K., Shinkai M., Honda H., Matsumoto K., Saida T., Kobayashi T. : Tumor regression by combined immunotherapy and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. Cancer Sci, 94 : 308-313, 2003.

44) Ito A., Shinkai M., Honda H., Kobayashi T. : Heat-inducible TNF-alpha gene therapy combined with hyperthermia using magnetic nanoparticles as a novel tumor-targeted therapy. Cancer Gene Ther, 8 : 649-654, 2001.

45) Ito A., Matsuoka M., Honda H., Kobayashi T. : Anititumor effects of combined therapy of recombinant heat shock protein 70 and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. Cancer Immunol Immunother, 53 : 26-32, 2004.

46) Ito A., Matsuoka M., Honda H., Kobayashi T. : Heat shock protein 70 gene therapy combined with hyperthermia using magnetic nanoparticles. Cancer Gene Ther, 10 : 918-925, 2003.

ハイパーサーミアによる腫瘍免疫賦活のメカニズム

井藤　彰・小林　猛・本多裕之

名古屋大学大学院工学研究科化学・生物工学専攻

要　　旨：近年，腫瘍免疫における熱ショック蛋白質 (HSP) の役割が注目されている．一方，HSP は温熱耐性を示す因子として，ハイパーサーミアにおいてはデメリットとして考えられてきた．もし，ハイパーサーミアにいける HSP 発現が腫瘍免疫賦活に関与するのなら，HSP 発現を積極的に利用するといった新しい概念に則った新しいガン免疫療法が開発できる．本稿では，ハイパーサーミアに伴う免疫賦活のメカニズムについて，HSP に注目して述べる．

Int. J. Cancer: **116,** 624–633 (2005)

Intratumoral injection of immature dendritic cells enhances antitumor effect of hyperthermia using magnetic nanoparticles

Kouji Tanaka[1], Akira Ito[1], Takeshi Kobayashi[1], Tatsuyoshi Kawamura[2], Shinji Shimada[2], Kazuhiko Matsumoto[3], Toshiaki Saida[3] and Hiroyuki Honda[1]*

[1]*Department of Biotechnology, School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Japan*
[2]*Department of Dermatology, School of Medicine, Yamanashi University, 1110, Shimogato, Tamah, Nakakoma, Japan*
[3]*Department of Dermatology, School of Medicine, Shinshu University, Asahi, Matsumoto, Nagano, Japan*

Dendritic cells (DCs) are potent antigen-presenting cells that play a pivotal role in regulating immune responses in cancer and have recently been shown to be activated by heat shock proteins (HSPs). We previously reported that HSP70 expression after hyperthermia induces antitumor immunity. Our hyperthermia system using magnetite cationic liposomes (MCLs) induced necrotic cell death that was correlated with HSP70 release. In the present study, we investigated the therapeutic effects of DC therapy combined with MCL-induced hyperthermia on mouse melanoma. In an *in vitro* study, when immature DCs were pulsed with mouse B16 melanoma cells heated at 43°C, major histocompatibility complex (MHC) class I/II, costimulatory molecules CD80/CD86 and CCR7 in the DCs were upregulated, thus resulting in DC maturation. C57BL/6 mice bearing a melanoma nodule were subjected to combination therapy using hyperthermia and DC immunotherapy *in vivo* by means of tumor-specific hyperthermia using MCLs and directly injected immature DCs. Mice were divided into 4 groups: group I (control), group II (hyperthermia), group III (DC therapy) and group IV (hyperthermia + DC therapy). Complete regression of tumors was observed in 60% of mice in group IV, while no tumor regression was seen among mice in the other groups. Increased cytotoxic T lymphocyte (CTL) and natural killer (NK) activity was observed on *in vitro* cytotoxicity assay using splenocytes in the cured mice treated with combination therapy, and the cured mice rejected a second challenge of B16 melanoma cells. This study has important implications for the application of MCL-induced hyperthermia plus DC therapy in patients with advanced malignancies as a novel cancer therapy.

Key words: hyperthermia; dendritic cell; antitumor immunity; magnetite cationic liposome; melanoma

Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients.[1] The most commonly used heating method in clinical settings is capacitive heating using a radiofrequency (RF) electric field.[2] However, the problem with capacitive heating using an RF electric field is the difficulty in heating only the local tumor region at the intended temperature without damaging normal tissue, because electromagnetic energy is conducted from outside the body by penetrating normal tissue and is imperfectly transduced to heat; the heating characteristics are influenced by various factors, such as tumor size, position of electrodes, and adhesion of electrodes at uneven sites. Magnetic nanoparticles have thus been applied to hyperthermia in an attempt to overcome these disadvantages.[3,4] Magnetic nanoparticles act as a transducer to change electromagnetic energy to heat; such nanoparticles generate heat in an alternating magnetic field (AMF) due to hysteresis loss. As a result, only tissue accumulating magnetite nanoparticles is heated.[5] We subsequently developed magnetite cationic liposomes (MCLs) for use as an intracellular heating mediator.[6] MCLs were developed to show improved adsorption and accumulation in tumor cells and have a 10-fold higher affinity for tumor cells than for neutrally charged magnetoliposomes due to electrostatic interaction with the negatively-charged cell membrane.[7] The hyperthermic effects of MCLs were examined in an *in vivo* study, and complete tumor regression was observed.[8,9] Furthermore, our hyperthermia system could be applied without any serious side effects to large tumors of greater than 15 mm in mice through the use of higher temperatures and frequent repeated treatment.[10]

We also reported that our hyperthermia system induced antitumor immunity.[11] Heat shock proteins (HSPs) are highly conserved proteins whose syntheses are induced by a variety of stresses, including heat stress.[12] Because expression of HSP70 protects cells from heat-induced apoptosis,[13] HSP70 expression is considered to be a complicating factor in hyperthermia. On the other hand, recent reports have shown the importance of HSPs, such as HSP70, HSP90 and glucose-regulated protein 96 (gp96), in immune reactions.[14,15] HSP-mediated antitumor immunity has been reported in human melanoma and HSP-peptide complexes purified from human melanoma cells induced a vaccine-like effect.[16] With regard to the mechanism of antitumor immunity induced by hyperthermia using MCLs, we previously demonstrated that HSP70 expression during hyperthermia-induced antitumor immunity against the T-9 rat glioma[17,18]; our hyperthermia system leads to vaccination with HSP70-peptide complexes via necrotic tumor cell death *in vivo*, thus resulting in antitumor immunity. This phenomenon, which may be designated *in situ* vaccination, has numerous implications for the development of novel antitumor therapies based on hyperthermia using MCLs. Furthermore, it has been revealed that HSPs play important roles in delivering antigens to antigen-presenting cells (APCs) and maturating dendritic cells (DCs) as danger signals.[19–22]

DCs are the most potent APCs and can induce an immune response to tumors via antigen uptake and maturation.[23] The efficient isolation and preparation of both human and murine DCs is now possible.[24,25] Therefore, a DC-based vaccine is a potential approach that could be used for treating malignant tumors. However, the use of mature DCs requires efficient methods to incorporate antigens into the DCs because mature DCs lose their ability to take up antigens. To date, several methods for induction of antitumor immunity by DCs have been investigated. The major methods for antigen loading into DCs are pulsing DCs with proteins or peptides extracted from tumor cells,[26–28] transfecting DCs with gene encoding tumor antigens,[29] fusing DCs with tumor cells[30,31] or culturing DCs with tumor cells.[32] Although some of these methods require primary culture of tumor cells from patients, primary cultures often fail to give the required number of cells, or expanded tumor cells often show different antigens from those expressed in tumor specimens due to changes in immunological characteristics over the long culture period for expansion. In addition, it is difficult to repeatedly obtain tumor specimens from patients with

Grant sponsor: Grants-in-Aid for Scientific Research, Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant number: 13853005; University Start-Ups Creation Support System, Ministry of Education, Culture, Sports, Science, and Technology of Japan; Grant sponsor: 21st Century COE Program "Nature-Guided Materials Processing," Ministry of Education, Culture, Sports, Science, and Technology of Japan.

***Correspondence to:** Department of Biotechnology, School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603 Japan. Fax: +81-52-789-3214. E-mail: honda@nubio.nagoya-u.ac.jp

Received 23 July 2004; Accepted after revision 13 January 2005
DOI 10.1002/ijc.21061
Published online 11 April 2005 in Wiley InterScience (www.interscience.wiley.com).

malignant cancer. One of the methods to overcome this problem is not to rely on antigen loading of DCs *in vitro* but on that *in situ* by intratumoral injection of immature DCs. It has been reported that intratumoral injection of DCs induced antitumor effects.[33,34] In addition, HSP70-enhanced antigen uptake[19] and HSP70-induced DC maturation[20,21] have been reported. This indicates that injection of DCs directly into tumors releasing HSP70 after hyperthermia using magnetite nanoparticles is a possible approach for immune induction.

In this study, we examined the influence of heated tumor cells on immature DCs and the feasibility of a novel combination therapy comprising hyperthermia with MCLs and DC therapy for malignant melanoma.

Material and methods

Cell lines and animal models

Mouse B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal calf serum (FBS), 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G. Mouse T-lymphoid EL4 cells were grown in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD), containing 10% FBS, 0.1 mg/ml streptomycin sulfate and 100 U/ml potassium penicillin G. Mouse YAC-1 cells were grown in RPMI 1640 medium, containing 10% FBS, 0.1 mg/ml streptomycin sulfate, 100 U/ml potassium penicillin G and 1.0 mM sodium pyruvate. Cells were grown at 37°C in an atmosphere containing 5% CO_2 and 95% air. Female C57BL/6 mice (age, 4 weeks) were purchased from Charles River Japan (Yokohama). To prepare tumor-bearing mice, 1×10^6 B16 melanoma cells were injected subcutaneously into the right flank of C57BL/6 mice, which were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight). Melanoma nodules that had grown to 6 mm in diameter were used for experiments. Tumor diameter was measured every 3 days and the average size was determined by applying the following formula, Tumor size = $0.5\times$(length + width)where length and width are measured in millimeters.

Animal experiments were performed according to the principles laid down in the "Guide for the Care and Use of Laboratory Animals" prepared under the direction of the Prime Minister of Japan.

Hyperthermic treatment of cultured cells

Hyperthermic treatment of cultured cells was performed as previously reported.[35] Briefly, B16 melanoma cells in the logarithmic growth phase were heated at 43°C for 30 min by direct immersion of cell culture dishes into a temperature-controlled water bath. The temperature of the medium increased quickly and reached 43°C within 5 min. The temperature in the medium was monitored with a fiber optic thermometer probe (FX-9020; Anritsu Meter, Tokyo, Japan).

Cell death assay

On the day following hyperthermic treatment, cell death was assayed by Vybrant Apoptosis Assay Kit (Molecular Probes, Eugene, OR). Cells were washed in PBS, after which the cell density was adjusted at 1×10^6 cells/ml in PBS. Cells were analyzed by flow cytometry (Paltec, Münster, Germany).

ELISA for HSP70 expression

After hyperthermic treatment, inducible HSP70 in the tumor cell extract was assayed using an HSP70 EIA Kit (StressGen Biotechnologies, British Columbia, Canada), which can detect and quantitate inducible HSP70 in samples originating from mice. ELISA was performed according to the manufacturer's instructions. The total protein content of the B16 melanoma cells was determined using the D_C protein assay reagent (Bio-Rad Laboratories, Hercules, CA).

Immature DC culture

Immature DCs were cultured from the bone marrow of C57BL/6 mice according to a protocol described by Inaba *et al.*[36] with slight modification. Briefly, murine bone marrow was harvested from the femur and tibia of sacrificed mice. To eliminate erythrocytes, dead cells and debris from murine bone marrow, cells were collected and suspended in RPMI 1640 medium, the same volume of Lympholyte-M (Sedarlane, Ontario, Canada) in the medium was underlaid, and this was centrifuged at 1,200*g* for 20 min at room temperature. After centrifugation, cells including DCs at the interface were collected and washed with medium. The cells were then plated in 100-mm cell culture dishes at a concentration of 10^7 cells in 10 ml of RPMI 1640 medium supplemented with 10 ng/ml recombinant mouse (rm) granulocyte macrophage colony stimulating factor (GM-CSF) (rmGM-CSF; Peprotech, Rocky Hill, NJ) and 10 ng/ml rm interleukin (IL)-4 (rmIL-4; Peprotech). Medium exchange was carried out every 2 days by gently swirling the plates, aspirating 75% of the medium, and adding fresh medium with rmGM-CSF and rmIL-4. On day 7, nonadherent and loosely adherent cells were collected and used as immature DCs.

Phenotypic assay of DCs

After *in vitro* hyperthermia, 5×10^6 heated or nonheated cells were collected by centrifugation at 1,200*g* for 5 min, and the cell pellets in 0.1 ml PBS were added to immature DCs and then cocultured for 48 hr. After the coculture, DCs were assayed for their phenotype. DCs were collected and washed in cold PBS, after which cell density was adjusted to 1×10^6 cells/ml in PBS. For flow cytometric analysis, DCs were washed twice with cold PBS containing 2% FBS and were then incubated for 40 min at 4°C with the fluorescein isthiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) (BD PharMingen, San Jose, CA) against murine cell-surface molecules H-$2K^b$, I-A^b, CD80, CD86 and isotype controls. Cells were washed 3 times with cold PBS and stained cells were analyzed by flow cytometry (Paltec).

Western blot analysis

CCR7 expression in DCs cocultured with heated or nonheated B16 cells was investigated by Western blot analysis. SDS samples of DCs (1×10^6) were heated at 100°C for 6 min and centrifuged at 18,000*g* for 5 min and supernatants were then subjected to SDS-PAGE on a 7.5% polyacrylamide gel. Protein blots were transferred to a nitrocellulose membrane, incubated with goat anti-CCR7 polyclonal antibodies (pAbs) for 1 hr at 37°C, probed with peroxidase-labeled rabbit anti-goat immunoglobulin G (IgG) for 1 hr at 37°C, and then visualized using an ECL (Amersham Pharmacia Biotech, Backinghamshire, UK).

Preparation of MCLs

MCLs were fabricated from a colloidal magnetite and lipid mixture consisting of *N*-(α-trimethylammonioacetyl)-didodecyl-D-glutamate chloride (Sogo Pharmaceutical, Tokyo, Japan), dilauroylphosphatidylcholine and dioleoylphosphatidyl-ethanolamine (Sigma Chemical, St. Louis, MO) in a 1:2:2 molar ratio, as described previously.[6]

In vivo *hyperthermia treatment and intratumoral injection of immature DCs*

Tumor-bearing mice were anesthetized with pentobarbital sodium (50 mg/kg intraperitoneally [i.p.]). Under anesthesia, a syringe (26-G needle) containing MCLs was inserted longitudinally into each tumor subcutaneously from the tumor edge. MCLs (net magnetite amount, 2 mg) were injected using an infusion pump (SP100i; World Precision Instruments, Sarasota, FL) for 30 min. Melanoma tumor-bearing mice were then separated into 4 groups. Mice in groups I (control) and III (DC therapy) were not subjected to AMF. After injection of MCLs, mice in groups II (hyperthermia) and IV (hyperthermia + DC therapy) were subjected to AMF for 30 min in a horizontal coil (inner diameter:

7 cm; length: 7 cm) with a transistor inverter (LTG-100-05; Daiichi High Frequency, Tokyo, Japan). Magnetic field frequency and intensity were 118 kHz and 384 Oe, respectively. Tumor and rectal temperatures were measured by optical fiber probe (FX-9020; Anritsu Meter). At 1 day after irradiation, the tumors of mice in groups III (DC therapy) and IV (hyperthermia + DC therapy) were injected with 2×10^6 immature DCs.

In vivo *migration assay of DCs*

Migration assay of DCs was carried out as follows. Immature DCs were incubated with 2×10^{-6} M PKH-2 (Sigma Chemical) at room temperature for 5 min and were rinsed twice with PBS according to the manufacturer's instructions.[37] Immature DCs stained with PKH-2 were injected into the tumors of mice treated with or without hyperthermia. After 24 hr, mice were killed and the inguinal lymph nodes were fixed in 10% formalin solution and were embedded in paraffin. Specimens were sectioned and DCs stained with PKH-2 were counted. For immunohistochemical staining of CCR7, these sections were incubated for 1 hr at 37°C with goat anti-CCR7 antibody (Abcam, Cambridge, UK). Subsequently, these sections were incubated for 1 hr at 37°C with phycoerythrin (PE)-conjugated rabbit anti-goat IgG (DAKO, Kyoto, Japan). The sections were examined using a fluorescence microscope (Olympus, Tokyo, Japan).

Assay for CTL activity

Splenic lymphocytes derived from cured mice, which were treated with hyperthermia plus DC therapy, at 14 days after combination therapy were isolated using the Medimachine System (Dako A/S, Glostrup, Denmark). Erythrocytes in the splenic lymphocytes were depleted with 0.75% ammonium chloride. Cytotoxic activity was determined by lactate dehydrogenase (LDH) release assay[38] according to the manufacturer's instructions (Promega, Madison, WI). B16 and EL4 cells were used as target cells. Briefly, target cells (5×10^4 cells) were mixed with different ratios of effector cells and cocultured for 5 hr at 37°C in an atmosphere containing 5% CO_2. Release of LDH was then evaluated. Percent cytotoxicity was calculated as follows:

$$\%\,\text{cytotoxicity} = [(\text{experimental} - \text{culture medium background})/(\text{maximum LDH release} - \text{culture medium background})] \times 100$$

Assay for natural killer cell activity

Splenic lymphocytes isolated using the Medimachine System (DAKO) were also used for assay of natural killer (NK) cell activity. Naive mice that had been born at almost the same time were used as controls. Cytotoxic activity of NK cells was determined by an LDH release assay[38] according to the manufacturer's instructions (Promega). NK-sensitive YAC1 cells were used as target cells. Briefly, target cells (5×10^4 cells) were mixed with different ratios of effector cells and cocultured for 5 hr incubation at 37°C in an atmosphere containing 5% CO_2.

Rechallenge of cured mice

Completely cured mice were challenged with B16 melanoma cells at 100 days after the first hyperthermic treatment. B16 cells (1×10^5 cells) were injected subcutaneously into the left flank of C57BL/6 mice. Also, T-lymphoid EL4 cells (1×10^5 cells), as irrelevant tumor cells, were challenged, in order to investigate whether the protective immune response showed tumor specificity. Tumor sizes were measured every 3 days, and the mice were judged to be tumor free when they did not bear palpable tumors (less than about 2 mm in diameter).

Statistical analysis

Levels of statistical significance in the migration assay were evaluated using the Mann-Whitney rank-sum test in WinSTAT (Light Stone International, Tokyo, Japan). For survival analysis, the differences in survival rates were analyzed by a log-rank test in WinSTAT.

Results

In vitro *hyperthermic effects on B16 melanoma cells*

The *in vitro* hyperthermic effects on morphology of cell death and HSP70 expression in B16 melanoma cells were investigated (Fig. 1). After heating at 43°C for 30 min, necrotic cells accounted for 27.5% of those observed while apoptotic cells were negligible. High HSP70 expression in viable cells (3.8 pg/cell) was also observed. In contrast, only 6.4% of cells were necrotic and very low levels of HSP70 expression were detected among nonheated cells.

Immature DCs pulsed with heated tumor cells

Mouse bone-marrow cells were cultured for 7 days in the presence of GM-CSF and IL-4, and nonadherent and loosely adherent cells were collected and used as immature dendritic cells. Cell surface phenotypes of DCs pulsed with heated tumor cells were then investigated. After *in vitro* heat treatment of B16 cells, 5×10^6 heated or nonheated cells were collected by centrifugation, and the cell pellets were added to immature DCs, and then cocultured for 48 hr. As shown in Figure 1*a*, since 27.5% of the heated tumor cells showed necrotic cell death, approximately 1.4×10^6 necrotic cells were estimated to be cocultured with immature DCs. The cell-surface phenotypes of DCs after the coculture are shown in Figure 2. Expression of MHC class I/II ($H\text{-}2K^b$/$I\text{-}A^b$) and costimulatory molecules, such as CD80/CD86, was upregulated in DCs after coculture with heated cells when compared with DCs after coculture with nonheated cells (Fig. 2*a*). Mean fluorescence intensity (MFI) of peaks for $H\text{-}2K^b$, $I\text{-}A^b$, CD80, CD86 and isotype control was 25 or 65, 25 or 75, 20 or 45, 15 or 40, and 4 in DCs cocultured with nonheated or heated B16 cells, respectively (Table I). The MFI of peaks in immature DCs without coculture were comparable to that of DCs cocultured with nonheated cells (data not shown). When immature DCs were cocultured with heated cells *in vitro*, the expression of chemokine receptor CCR7, which is upregulated on mature DCs and leads them to regional lymph nodes, was investigated. It was found that CCR7 was strongly expressed in DCs when compared with DCs pulsed with nonheated cells or nonpulsed DCs (Fig. 2*b*). These results indicate that heated tumor cells induce DC maturation *in vitro*.

In vivo *hyperthermic treatment using MCLs*

For hyperthermic treatment *in vivo*, MCLs were injected into the tumor and an AMF was applied to the whole body of the mouse. Figure 3 shows the temperature at the tumor surface and in the rectum during AMF irradiation. Tumor temperature increased rapidly to 43°C in 5 min and was then maintained for 30 min by controlling AMF power. In contrast, the temperature in the rectum hardly increased during irradiation. This suggests that our hyperthermia system using MCLs is able to heat the tumor specifically without damaging healthy tissue. The tumor temperature was also maintained very precisely, thus demonstrating the ease of temperature control within the tumor by adjusting the power of the AMF generator.

Migration of DCs to the lymph nodes

DC migration from the tumor site to regional lymph nodes after intratumoral injection was examined. Immature DCs stained with PKH-2 were injected into the tumors treated with MCL-induced hyperthermia. At 24 hr after intratumoral injection, significantly larger numbers of labeled cells were observed in the lymph nodes of mice treated with hyperthermia than those of the untreated mice

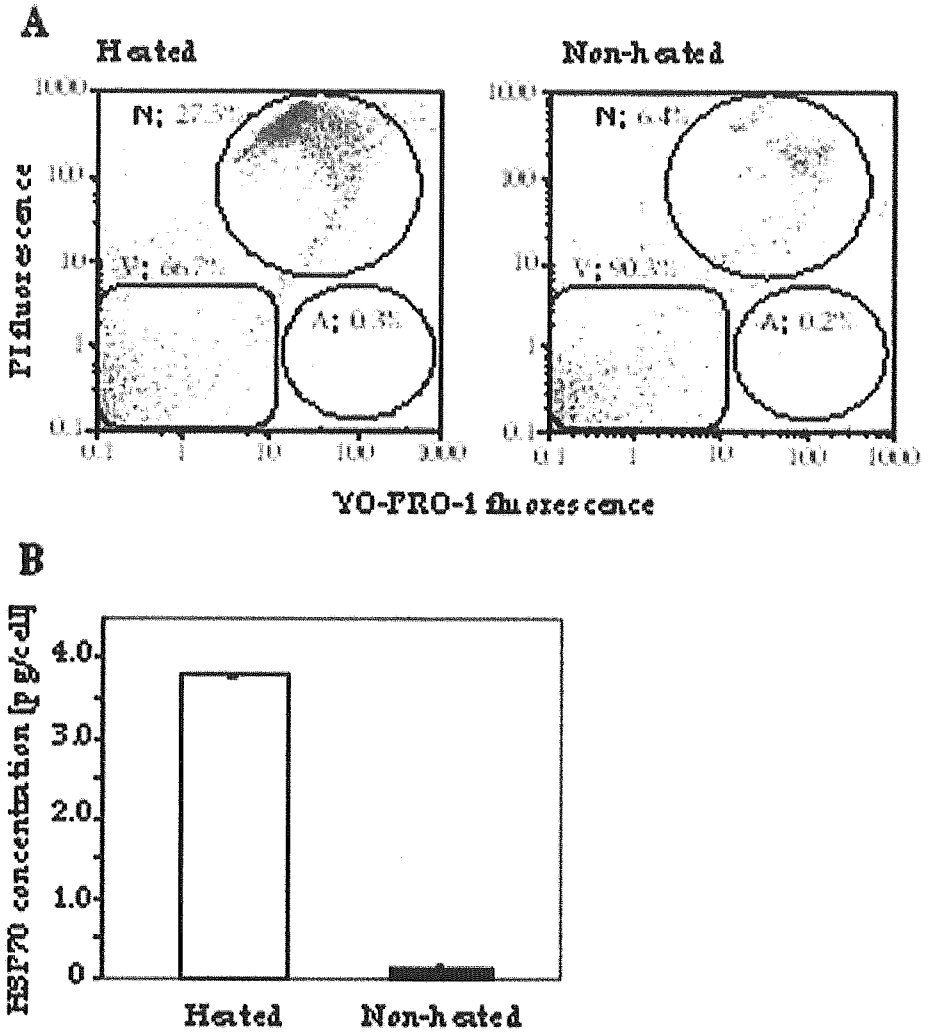

FIGURE 1 – Necrotic cell death and HSP70 expression after hyperthermia. B16 melanoma cells were subjected to hyperthermia at 43°C for 30 min *in vitro*. *(a)* Necrosis detection assay by flow cytometry of the cells 1 day after hyperthermic treatment (left) and nonheated cells (right). V: viable cells, N: necrotic cells, A: apoptotic cells. *(b)* Inducible HSP70 assay using ELISA in heated tumor cells (open bar) and nonheated tumor cells (closed bar). Data points represent mean ± standard deviation (SD) of 3 independent experiments.

(Fig. 4*a*, *b* and *c*). Furthermore, the double staining of DCs using PKH-2 (cell tracking marker) and anti-CCR7 antibody revealed that the DCs, which migrated into the lymph node, expressed CCR7 (Fig. 4*d*, *e* and *f*), suggesting that heated tumor cells activate the ability of DC migration into the lymph node *in vivo*.

Therapeutic effects of hyperthermia plus DC therapy on melanoma

The therapeutic effects of MCL-induced hyperthermia with immature DC therapy were assessed *in vivo*. Figure 5*a* shows the time courses of tumor growth. After melanoma nodules had grown to 6 mm in diameter, hyperthermia plus DC therapy was carried out. In group I (control), tumors grew progressively. In group II, in which mice received only hyperthermic treatment at 43°C, tumors grew progressively. Although we previously reported that 2 rounds of hyperthermia at 46°C could induce complete regression of B16 melanoma nodules,[9] in this study AMF was carried out once at 43°C in order to investigate the combined effects of MCL-induced hyperthermia plus immature DCs. When mice were treated with immature DCs (group III), tumor growth in 2 of 10 mice was suppressed but no complete regression was observed. On the other hand, when MCL-induced hyperthermia was combined with immature DC injection (group IV), tumor growth was apparently suppressed and 60% (6/10) of subcutaneous tumors regressed completely.

The survival rates of tumor-bearing mice over a period of 100 days after injection of MCLs are shown in Figure 5*b*. In groups I and II, all mice died from pulmonary metastases and/or an enlarged tumor at the inoculated site within 40 days. Survival was slightly prolonged in group III (DC therapy), and 1 of 10 mice survived for 60 days. In group IV (hyperthermia + DC therapy), 6 tumors regressed completely, as shown in Figure 5*a*, and did not begin growing again, while those mice showing regression (60%) survived for 100 days. A significant prolonging of survival in the combination group was observed when compared with the other groups ($p < 0.05$ *vs.* all other groups). These results indicate that survival of melanoma-burdened mice was prolonged by combination therapy comprising hyperthermia with MCLs and injection of immature DCs.

Antitumor immunity in hyperthermia plus DC therapy

An *in vitro* cytotoxicity assay was performed to examine whether the combined therapy induced antitumor immunity. Splenic lymphocytes were derived from cured mice treated with MCL-induced hyperthermia plus immature DCs (in group IV), and CTL assay was carried out. As shown in Figure 6*a*, splenic lymphocytes showed cytotoxic activity against B16 melanoma

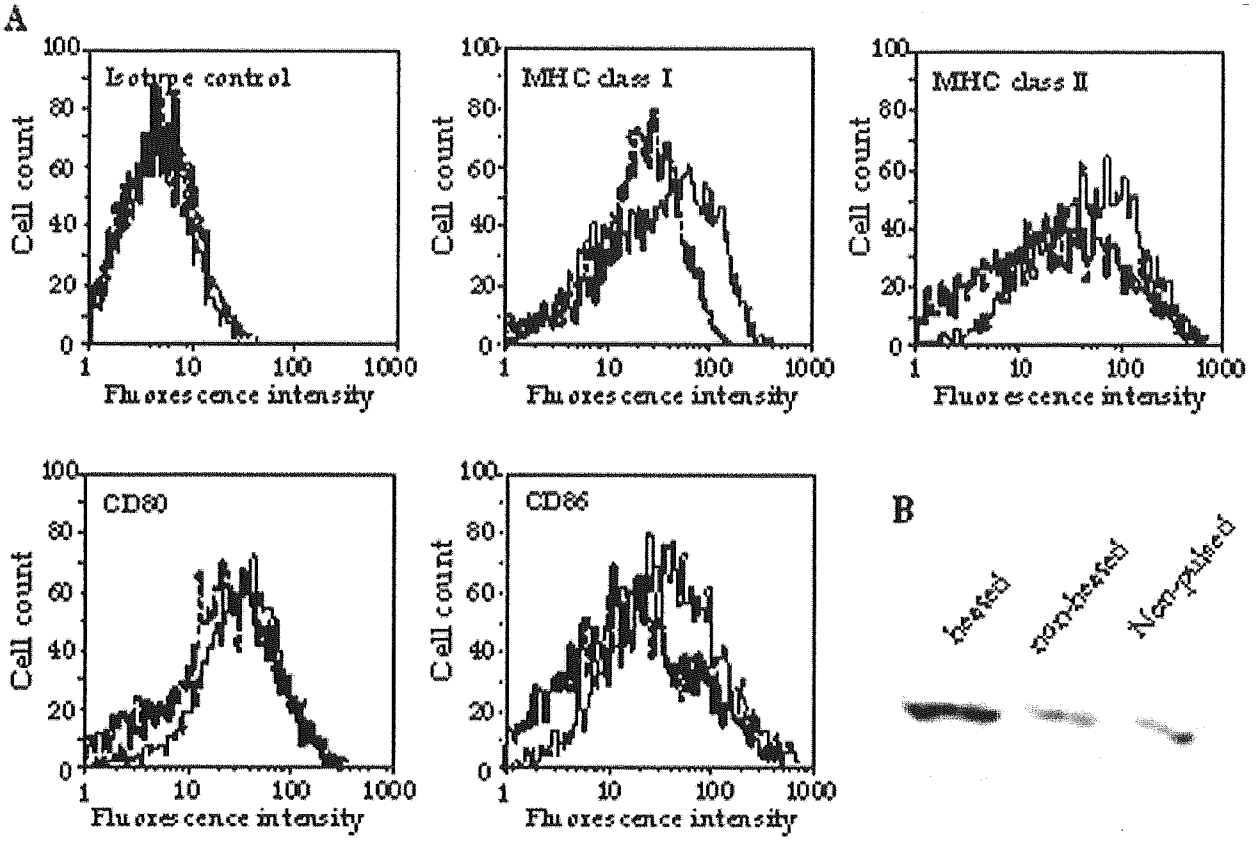

FIGURE 2 – DC phenotype after coculture with heated cells. Bone marrow-derived immature DCs were cocultured with heated B16 cells or nonheated B16 cells. After 48 hr, DCs were collected and their phenotypes were analyzed. *(a)* Flow cytometry analysis using FITC-conjugated mAbs against MHC class I/II, CD80, CD86 and isotype control. Solid line: pulsed with heated cells, dotted line: pulsed with nonheated cells. *(b)* CCR7 assay by Western blotting. CCR7 expression of the DCs (1×10^6) was determined as described in Material and methods.

TABLE I – MEAN FLUORESCENCE INTENSITY (MFI) OF DC SURFACE PROTEINS AFTER CO-CULTURE WITH HEATED OR NON-HEATED B16 CELLS[1]

	MHC class I	MHC class II	CD11c	CD80	CD86	Isotype control
Heated	65	75	30	45	40	4
Non-heated	25	25	20	20	15	4

[1]Bone marrow-derived immature DCs were co-cultured with heated B16 cells or non-heated B16 cells. After 48 hr DCs were collected and their phenotypes were analyzed by flow cytometer.

cells, and this activity was higher than that against EL4 cells (control). For NK activity, splenic lymphocytes were derived from cured mice (in group IV) and naive mice. NK-sensitive YAC1 cells were used as target cells. NK activity of the spleen cells derived from cured mice was also higher than that of spleen cells from naive mice (Fig. 6*b*). These results indicate that both CTLs and NK cells were induced by hyperthermia plus DC therapy. Therefore, in order to confirm that the antitumor immunity could reject B16 melanoma *in vivo*, mice exhibiting complete regression after combination therapy were challenged with 1×10^5 B16 cells at day 100 after treatment. As shown in Figure 7*a*, all naive mice subsequently formed tumors within 10 days. In contrast, all cured mice rejected the B16 cells and no tumors appeared in these mice for up to 30 days after inoculation (Fig. 7*a*). Also, when T-lymphoid EL4 cells as irrelevant tumor cells were challenged in the cured mice, none of them rejected the EL4, suggesting that the protective immune response was tumor specific (Fig. 7*b*).

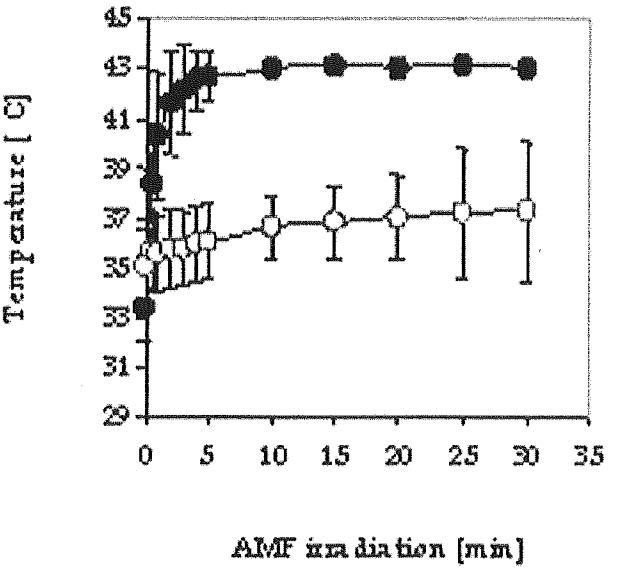

FIGURE 3 – MCL-induced hyperthermia *in vivo*. MCLs were injected directly into subcutaneous B16 tumors of mice, which were then irradiated with an alternating magnetic field (AMF) for 30 min. Tumor and rectal temperatures were measured by optical fiber probes. Closed circles: tumor; open circles: rectum. Each point represents the mean ± standard deviation (SD) of 10 mice.

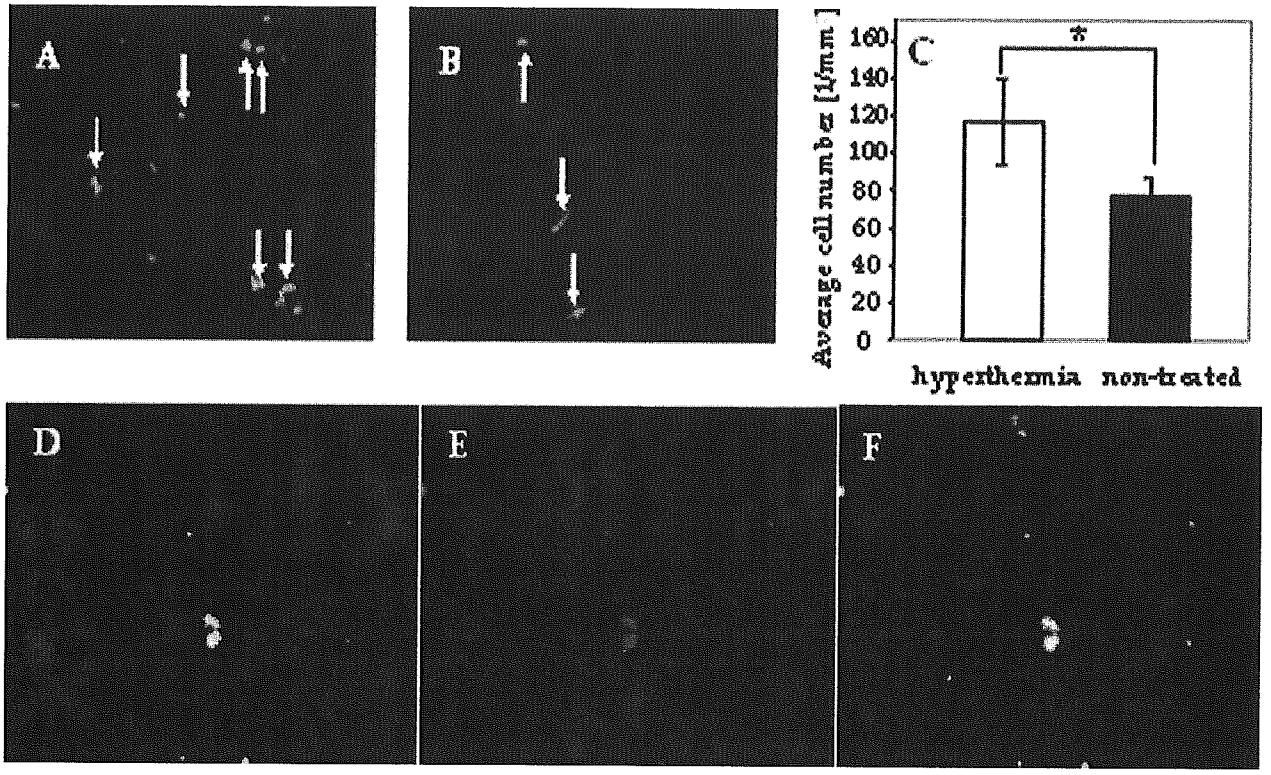

FIGURE 4 – Migration of DCs to lymph nodes. PKH2-stained immature DCs were injected into tumors treated with MCL-induced hyperthermia. At 24 hr after intratumoral injection of DCs, mice were dissected and inguinal lymph nodes were extirpated. Photographs of lymph node specimens of the mice treated with *(a)* or without *(b)* hyperthermia. The arrows indicate fluorescent cells. Positive cells were counted and averaged over 3 1-mm^2 sections *(c)*. Data represents mean ± standard deviation (SD) of 3 independent experiments. *$p < 0.05$. The migrated DCs into lymph nodes were subsequently immunohistochemically stained with anti-CCR7 polyclonal antibody. Green cells in *(d)* are DCs that were prelabeled with PKH2, red cells in *(e)* are CCR7-positive DCs, and *(f)* is the merged photograph *(d,e)* and yellow cells are both PKH2- and αCCR7-positive DCs.

Discussion

We previously reported that hyperthermia using MCLs killed tumors not only by heat but also by immune induction. In a previous study, we reported the infiltration of CD8-positive T cells and Mac-3-positive macrophage/DCs into a tumor and the activation of cytotoxicity against a melanoma nodule after hyperthrmia.[39] Our hyperthermia system using MCLs and AMF induced antitumor immunity, but other cancer therapies, such as chemotherapy and radiotherapy, often decrease leukocytes and surgery also causes attenuation of immunity via hyposthenia. Our hyperthermia system is able to heat the tumor specifically by means of the MCLs (Fig. 3), and the degree of heat generation in the tumor is controlled by the MFI, which allows the induction of necrotic cell killing without damaging the surrounding normal tissues. Because nontoxic therapy is effective for immune induction, the toxicity of MCLs should be carefully investigated. In our preliminary studies, the toxicity of a single administration of MCL solution (33 mg of magnetite, i.p.) was investigated; MCLs largely accumulated in the liver and spleen of mice, but none of the 5 observed mice died after MCL injection.[40] Although the amount of MCLs used in this study (2 mg of magnetite) was much smaller, MCL toxicity should be fully investigated before clinical application.

We previously demonstrated 2 mechanisms of antitumor immunity in MCL-induced hyperthermia: *(i)* augmentation of antigenicity of T-9 rat glioma cells by increasing MHC class I expression on cell surface via HSP70 expression[17] and *(ii)* necrotic cell killing via HSP70 release, which caused *in situ* vaccination.[18] We thus demonstrated that expression of HSP70 was involved in antitumor immunity induced by hyperthermia. We previously reported that MCL-induced hyperthermia treatment at 43°C for 30 min induced necrotic cell death in B16 melanoma.[39] In this study, apoptosis detection assay by the terminal deoxynucreotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method was performed, but no significant number of apoptotic cells was observed (data not shown). On the other hand, conventional hyperthermia systems are applied to induce apoptosis, not necrotic cell death. This is because lower temperatures are used to minimize damage to the surrounding normal tissues due to lack of heating specificity. Yonezawa *et al.*[41] compared the manner of cell death induced by hyperthermia; apoptotic cell death was induced in malignant fibrous histiocytoma cells by hyperthermia treatment at 42°C, while necrotic cell death was induced when the cells were heated at higher temperatures (44°C). Although the temperature inducing apoptosis or necrosis may differ between cell types, higher temperatures should result in necrotic cell death. For B16 melanoma cells, necrotic cell death was induced by heating at 43°C for 1 hr (Fig. 1*a*). Accompanying the heat stress that caused necrotic cell death, B16 melanoma cells expressed inducible HSP70 (Fig. 1*b*). Because expression of HSP70 protects cells from heat-induced apoptosis,[13] HSP70 expression is considered to be a complicating factor in hyperthermia. However, there have been several reports that HSP70 expression could induce strong antitumor immunity.[42]

It has been shown that HSPs play an important role in carrying antigens to APCs and in the maturation of DCs by acting as a danger signal. DCs are potent APCs and can regulate immune responses in cancer. Cancer therapy is currently focused on the

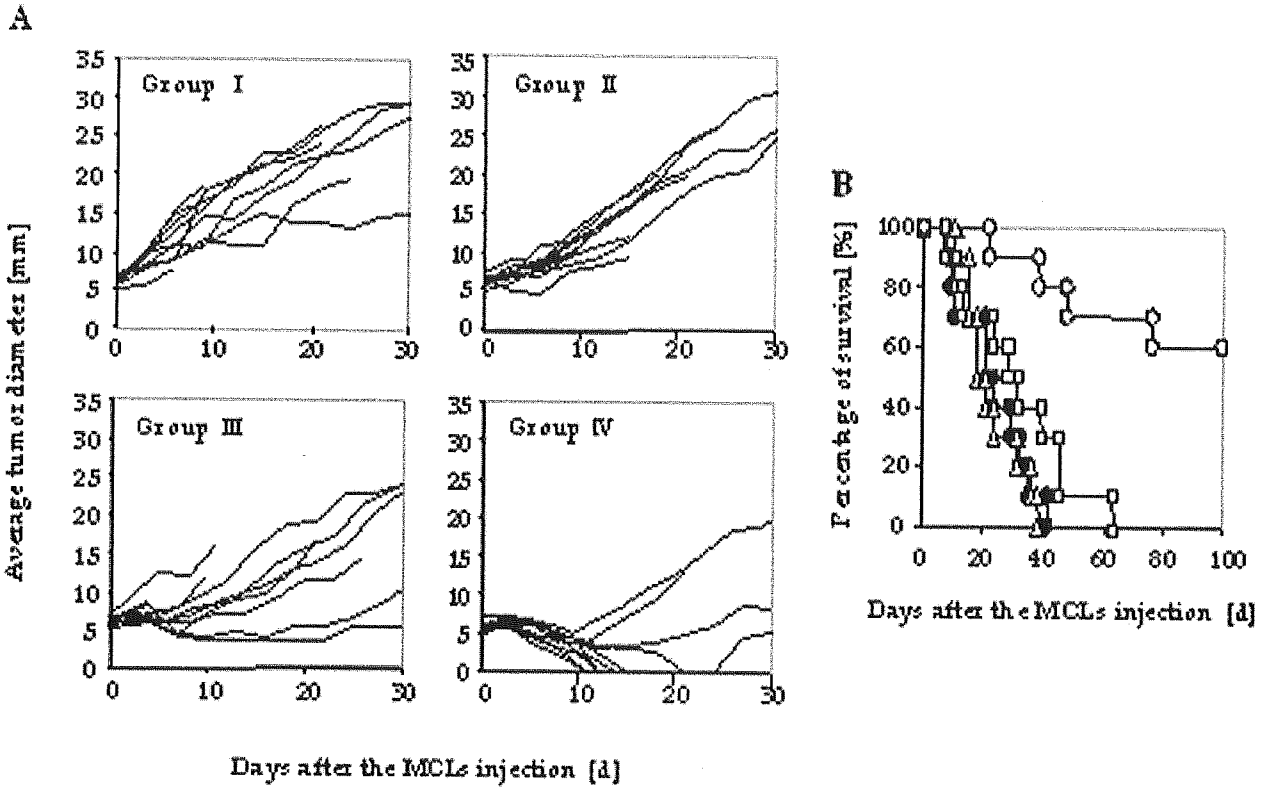

FIGURE 5 – Antitumor effects of combination therapy of MCL-induced hyperthermia with immature DC therapy. Mice in each group were treated as described in Material and methods. *(a)* Time course of tumor growth. Group I (control, $n = 10$), group II (hyperthermia, $n = 10$), group III (DC therapy, $n = 10$) and group IV (hyperthermia + DC therapy, $n = 10$). Each line represents tumor growth kinetics in a single mouse. *(b)* Percentage survival of tumor-bearing mice. Closed circles, group I (control); open triangles, group II (hyperthermia); open squares, group III (DC therapy) and open circles, group IV (hyperthermia + DC therapy). Each group included 10 mice. Survival in group IV was significantly prolonged when compared with the other groups ($p < 0.05$ *vs.* all other groups).

application of DCs to vaccination and cancer immunotherapy. Antigenic peptides of tumor antigens are presented by MHC molecules on DCs with costimulatory molecules, such as CD80/CD86, and are recognized by specific T cell receptors. For antigen-presentation by DCs, the uptake of tumor antigens by DCs and their maturation is necessary. Todryk *et al.*[19] showed that HSP70 chaperoning antigens, which are released from dying tumor cells, are directly taken up by DCs and enhance antigen uptake. Furthermore, Basu *et al.*[21] reported that necrotic, but not apoptotic, cell death leads to the release of biologically potent HSPs from cells, which deliver a partial maturation signals to DCs. In this study, heated B16 cells induced the maturation of DCs, which enhanced antigen presentation and migration to lymph nodes, as shown in Figures 2 and 4. Figure 2 shows that MHC molecules ($H\text{-}2K^b$, $I\text{-}A^b$) and costimulatory molecules (CD80, CD86) were upregulated in DCs cocultured with heat-treated B16 cells, which suggests that DCs matured and their antigen-presenting ability was increased. In addition, CCR7 was upregulated by coculture with heated B16 cells (Fig. 2*b*). DC migration is believed to be one of the most important functions of mature DCs in the afferent limb of the immune response, and CCR7 plays an important role in DC migration to lymph nodes.[43] After intratumoral injection, significantly larger numbers of labeled cells were observed in the lymph nodes of mice treated with hyperthermia when compared with untreated mice, as shown in Figure 4, and these migrating DCs expressed CCR7. These results suggest that our hyperthermia system induced a vaccine-like effect caused by necrotic cell death via HSP70 expression *in vivo* and that its combination with immature DCs was a potent therapeutic methodology.

The observed DC maturation caused by heated cells prompted us to inject immature DCs into the tumor after MCL-induced hyperthermia, and we investigated the therapeutic effects of MCL-induced hyperthermia plus immature DC therapy. Although hyperthermia at 43°C once was insufficient to cause tumor regression, 60% (6/10) of tumors in the mice of group IV (hyperthermia + DC therapy) were completely regressed and cured mice survived for over 100 days (Fig. 5), thus suggesting that the therapeutic effects of MCL-induced hyperthermia were enhanced by injection of immature DCs. In some mice in the DC therapy group (group III), tumor growth was inhibited, but survival was largely the same as in the control group, with many of the dead mice exhibiting metastasis to the lung (data not shown), which suggests that injection of immature DCs alone was not able to elicit sufficient immunity for rejection of metastasis. On the other hand, all cured mice in group IV rejected a second challenge of B16 cells on the opposite side of the first challenge (Fig. 7*a*), which suggests that systemic antitumor immunity was acquired and metastasis was inhibited by hyperthermia plus DC therapy.

The present study revealed that both CTLs and NK cells were activated in mice treated with combination therapy (Fig. 6). DCs are known to be able to prime naive T cells by presenting tumor antigens via MHC class I/II. B16 mouse melanoma cells are known to be poorly immunogenic because they express a low amount of H-2 antigen, and widely utilized in immunological experiments to assess the effect of immunotherapy. We previously studied the $H\text{-}2K^b$ amount on B16 cells by flow cytometry.[39] Flow cytometric analysis using the anti-mouse $H\text{-}2K^b$ antibody revealed that B16 cells used in this study expressed a much lower amount

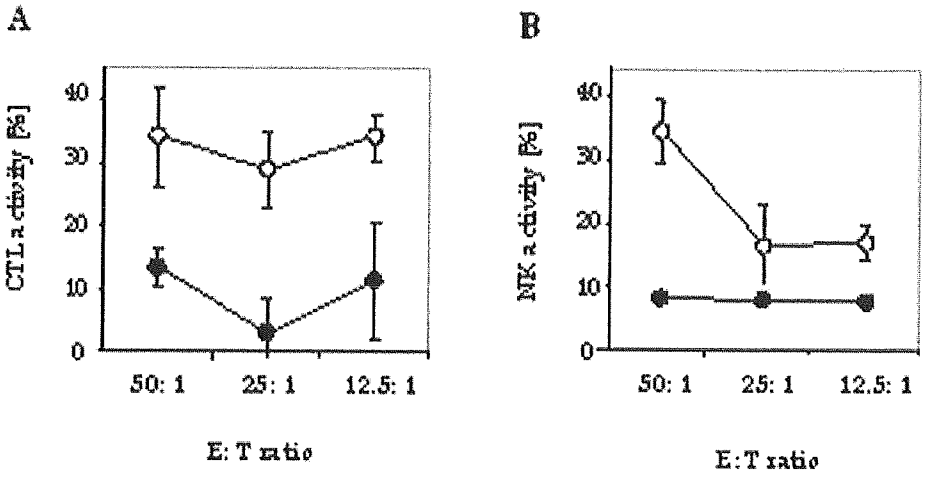

FIGURE 6 – Cytotoxic activity of splenocytes after combination therapy of MCL-induced hyperthermia with DC therapy. *(a)* CTL assay against B16 melanoma cells. Spleen cells were derived from cured mice in the hyperthermia plus DC therapy group at 14 days after combination therapy. B16 (open circles) and EL4 (closed circles) were used as target cells. *(b)* NK assay. Spleen cells were derived from cured mice in the hyperthermia plus DC therapy group at 14 days after combination therapy (open circles) and from naive mice (closed circles). NK-sensitive YAC1 cells were used as targets. Effector cell:target cell (E:T) ratios ranged from 50:1 to 12.5:1. Data points represent mean ± SD of 3 experiments.

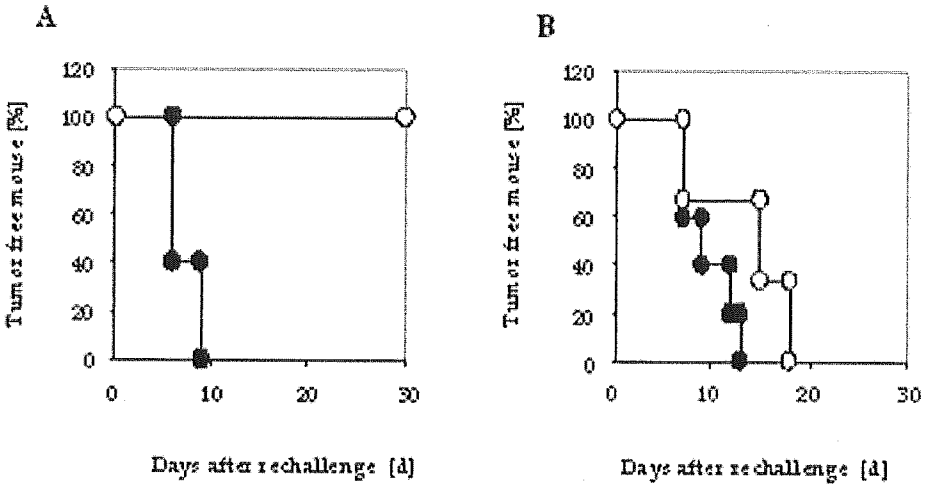

FIGURE 7 – *(a)* Time course of percent tumor-free mouse after rechallenge of B16 cells at 100 days after combination therapy. *(b)* Time course of percent tumor-free mouse after challenge of EL4 cells at 30 days after combination therapy. Mice cured by combined therapy comprising MCL-induced hyperthermia with immature DC therapy (open circles) and naive mice (closed circles) were challenged with B16 or EL4 cells.

of MHC class I antigen when compared with EL4 cells. In this study, we used poorly immunogenic B16 cells to assess the preclinical feasibility of a novel cancer therapy. Efficient recognition of tumor cells by CTLs is often dependent on the presentation of cytosolic peptides in the context of MHC class I molecules. This process may be influenced by various molecular chaperones, such as HSPs. Wells *et al.*[43] reported that stably transfected clones of B16 that constitutively expressed human HSP70 exhibited significantly increased surface levels of MHC class I antigen. This HSP70-mediated upregulation of surface MHC class I antigen represents increases in both the amount of functional MHC-peptide complex, as measured by conformation-dependent antibodies and the recognition by MHC class I-restricted CTLs. We previously demonstrated the augmentation of MHC class I antigen presentation via HSP70 expression after hyperthermia.[17,39] The T-9 rat glioma cell surface presentation of MHC class I antigen increased in tandem with increased HSP70 expression and the immunogenicity of these cells was enhanced by hyperthermia,[17] which indicates a mechanism of antitumor immunity induced by hyperthermia. In addition, DCs have been reported to activate NK cells by secretion of cytokines (IL-12 and IL-18) and costimulatory molecules.[45,46] Because NK cells can kill cancer cells that show low MHC class I expression, the activation of not only CTLs but also NK cells is helpful in melanoma therapy. These cytotoxic cells are stimulated in the lymph nodes by antigen presentation and/or cytokine secretion of mature DCs that migrate after hyperthermia. These effector cells then contribute to tumor regression and inhibition of metastasis in mice subjected to combination therapy. Although further study is needed to elucidate the dominant effector cells (CTL or NK cell) in melanoma therapy, the antitumor immunity induced by this combination therapy may be effective in all tumors.

In summary, we observed that heated tumor cells induced DC maturation and that MCL-induced hyperthermia plus immature DC therapy induced antitumor immunity. We previously reported that higher temperatures and frequent treatment using our hyperthermia system caused strong antitumor effects, not only directly on the tumor but also via systemic immune response.[9] Udono *et al.*[47] reported that the HSP70 vaccination effect was directly dependent on the dose of HSP70. Because intense hyperthermia induces larger amounts of necrotic cell death and HSP expression, our hyperthermia system may strongly promote the maturation of

DCs as part of an *in situ* vaccination. Based on these results, we believe that combination therapy of hyperthermia using MCLs and intratumoral injection of immature DCs is applicable to patients with advanced malignancies as a novel cancer therapy.

Acknowledgements

We thank the Toda Kogyo Company (Hiroshima, Japan) for supplying the magnetite.

References

1. van der Zee J. Heating the patient: a promising approach? Ann Oncol 2002;13:1173–84.
2. Abe M, Hiraoka M, Takahashi M, Egawa S, Matsuda C, Onoyama Y, Morita K, Kakehi M, Sugahara T. Multi-institutional studies on hyperthermia using an 8-MHz radiofrequency capacitive heating device (Thermotron RF-8) in combination with radiation for cancer therapy. Cancer 1986;58:1589–95.
3. Jordan A, Wust P, Fahling H, John W, Hinz A, Felix R. Inductive heating of ferrimagnetic particles and magnetic fluids: physical evaluation of their potential for hyperthermia. Int J Hyperthermia 1993;9:51–68.
4. Minamimura T, Sato H, Kasaoka S, Saito T, Ishizawa S, Takemori S, Tazawa K, Tsukada K. Tumor regression by inductive hyperthermia combined with hepatic embolization using dextran magnetite-incorporated microspheres in rats. Int J Oncol 2000;16:1153–8.
5. Shinkai M, Matsui M, Kobayashi T. Heat properties of magnetoliposomes for local hyperthermia. Jpn J Hyperthermic Oncol 1994;10: 168–77.
6. Shinkai M, Yanase M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study. Jpn J Cancer Res 1996;87:1179–83.
7. Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposomes: ex vivo study. Jpn J Cancer Res 1997;88: 630–2.
8. Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using magnetite cationic liposome: an in vivo study. Jpn J Cancer Res 1998;89:463–9.
9. Suzuki M, Shinkai M, Honda H, Kobayashi T. Anti-cancer effect and immune induction by hyperthermia of malignant melanoma using magnetite cationic liposomes. Melanoma Res 2003;13:129–35.
10. Ito A, Tanaka K, Honda H, Abe S, Yamaguchi H, Kobayashi T. Complete regression of mouse mammary carcinoma with a size greater than 15 mm by frequent repeated hyperthermia using magnetite nanoparticles. J Biosci Bioeng 2003;96:364–9.
11. Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Antitumor immunity induction by intracellular hyperthermia using magnetite cationic liposomes. Jpn J Cancer Res 1998;89:775–82.
12. Schlesinger MJ. Heat shock proteins. J Biol Chem 1990;265:12111–4.
13. Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, Massie B. The chaperon function of hsp 70 is required for protection against stress-induced apoptosis. Mol Cell Biol 2000; 20:7146–59.
14. Srivastava PK, Menoret A, Basu S, Binder R, Quade K. Heat shock proteins come of age: primitive functions acquired new roles in an adaptive world. Immunity 1998;8:657–65.
15. Menoret A, Chandawarkar R. Heat-shock protein-based anticancer immunotherapy: An idea whose time has come. Semin Immunol 1998;25:654–60.
16. Castelli C, Ciupitu AM, Rini F, Rivoltini L, Mazzocchi A, Kiessling R, Parmiani G. Human heat shock protein 70 peptide complexes specifically activate antimelanoma T cells. Cancer Res 2001;61: 222–7.
17. Ito A, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Augmentation of MHC class I antigen presentation via heat shock protein expression by hyperthermia. Cancer Immunol Immunother 2001;50:515–22.
18. Ito A, Shinkai M, Honda H, Yoshikawa K, Saga S, Wakabayashi T, Yoshida J, Kobayashi T. Heat shock protein 70 expression induces antitumor immunity during intracellular hyperthermia using magnetite nanoparticles. Cancer Immunol Immunother 2003;52:80–8.
19. Todryk S, Melcher AA, Hardwick N, Linardakis E, Bateman A, Colombo MP, Stoppacciaro A, Vile RG. Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. J Immunol 1999;163:1398–1408.
20. Noessoner E, Gastpar R, Milani V, Brandl A, Hutzler RJS, Kuppner MC, Roos M, Kremmer E, Asea A, Calderwood SK, Issels RD. Tumor-derived heat shock protein 70 peptide complexes are cross-presented by human dendritic cells. J Immunol 2002;169: 5424–32.
21. Basu S, Binder RJ, Suto R, Anderson KM, Srivastava PK. Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kB pathway. Int Immunol 2000;12:1539–46.
22. Campici J, Leem TD, Fleshner M. Stress-induced extracellular Hsp72 is a functionally significant danger signal to the immune system. Cell Stress Chaperones 2003;8:272–86.
23. Winzler C, Rovere P, Rescigno M, Granucci F, Penna G, Adorini L, Zimmermann VS, Davoust J, Ricciardi-Castagnoli P. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med 1997;20:317–28.
24. Nestle FO, Alijagic S, Gilliet M, Sun Y, Grabbe S, Dummer R, Burg G, Schadendorf D. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 1998;68:328–32.
25. Reeves ME, Royal RE, Lam JS, Rosenberg SA, Hwu P. Retroviral transduction of human dendritic cells with a tumor-associated antigen gene. Cancer Res 1996;56:5672–7.
26. Nair SK, Snyder D, Rouse BT, Gilboa E. Regression of tumors in mice vaccinated with professional antigen-presenting cells pulsed with tumor extracts. Int J Cancer 1997;70:706–15.
27. Tjandrawan T, Martin DM, Maeurer MJ, Castelli C, Lotze MT, Storkus WJ. Autologous human dendriphages pulsed with synthetic or natural tumor peptides elicit tumor-specific CTLs in vitro. J Immunother 1998;21:149–57.
28. Zitvogel L, Mayordomo JI, Tjandrawan T, DeLeo AB, Clarke MR, Lotze MT, Storkus WJ. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. J Exp Med 1997; 183:87–97.
29. Tuting T, Wilson CC, Martin DM, Kasamon YL, Rowles J, Ma DI, Slingluff CL, Wagner SN, Van der Bruggen P, Baar J, Lotze MT, Storkus WJ. Autologous human monocyte-derived dendritic cells genetically modified to express melanoma antigens elicit primary cytotoxic T cell responses in vitro: enhancement by cotransfection of genes encoding the Th1-biasing cytokines IL-12 and IFN-alpha. J Immunol 1998;160:1139–47.
30. Akasaki Y, Kikuchi T, Homma S, Abe T, Kufe D, Ohno T. Antitumor effect of immunizations with fusions of dendritic and glioma cells in a mouse brain tumor model. J Immunother 2001; 24:106–13.
31. Homma S, Toda G, Gong J, Kufe D, Ohno T. Preventive antitumor activity against hepatocellular carcinoma (HCC) induced by immunization with fusions of dendritic cells and HCC cells in mice. J Gastroenterol 2001;36:764–71.
32. Celluzzi CM, Falo LD. Physical interaction between dendritic cells and tumor cells results in an immunogen that induces protective and therapeutic tumor rejection. J. Immunol 1998;160: 3081–5.
33. Nishioka Y, Hirao M, Robbins PD, Lotze MT, Tahara H. Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin-12. Cancer Res 1999;59:4035–41.
34. Kikuchi T, Miyazawa N, Moore MA, Crystal RG. Tumor regression induced by intratumor administration of adenovirus vector expressing CD40 ligand and naive dendritic cells. Cancer Res 2000;60: 6391–5.
35. Ito A, Shinkai M, Bouhon IA, Honda H, Kobayashi T. Induction of *TNF-α* gene expression by heat inducible promoter *gadd* 153. Jpn J Hyperthermic Oncol 2000;16:91–6.
36. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM. Generation of large Numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 1992;176: 1693–702.
37. Kikuchi T, Akasaki Y, Abe T, Ohno T. Intratumoral injection of dendritic and irradiated glioma cells induces anti-tumor effects in a mouse brain tumor model. Cancer Immunol Immunother 2002;51: 424–30.
38. Chen CH, Wang TL, Hung CF, Yang Y, Young RA, Pardoll DM, Wu TC. Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. Cancer Res 2000;60:1035–42.
39. Ito A, Tanaka K, Kondo M, Shinkai M, Honda H, Matsumoto K, Saida T, Kobayashi T. Tumor regression by combined immunotherapy and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. Cancer Sci 2003;94: 308–13.
40. Ito A, Nakahara Y, Tanaka K, Kuga Y, Honda H, Kobayashi T. Time course of biodistribution and heat generation of magnetite cationic liposomes in mouse model. Jpn J Hyperthermic Oncol 2003;19: 151–59.

41. Yonezawa M, Ohtsuka T, Matsui N, Tsuji H, Kato KH, Moriyama A, Kato T. Hyperthermia induces apoptosis in malignant fibrous histiocytoma cells in vitro. Int J Cancer 1996;66:347–51.
42. Melcher A, Todryk S, Hardwick N, Ford M, Jacobson M, Vile MJ. Tumor immunogenicity is determined by the mechanism of cell death via induction of heat shock protein expression. Nat Med 1998; 4:581–7.
43. Wells AD, Rai SK, Salvato MS, Band H, Malkovsky M. Hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. Int Immunol 1998;10: 609–17.
44. Hirao M, Onai N, Hiroishi K, Watkins SC, Matsushima K, Robbins PD, Lotze MT, Tahara H. CC chemokine receptor-7 on dendritic cells is induced after interaction with apoptotic tumor cells: critical role in migration from the tumor site to draining lymph nodes. Cancer Res 2000;60:2209–17.
45. Geldhof AB, Moser M, Lespagnard L, Thielemans K, De Baetselier P. Interleukin-12-activated natural killer cells recognize B7 costimulatory molecules on tumor cells and autologous dendritic cells. Blood 1998;91:196–206.
46. Yu Y, Hagihara M, Ando K, Gansuvd B, Matsuzawa H, Tsuchiya T, Ueda Y, Inoue H, Hotta T, Kato S. Enhancement of human cord blood CD34+ cell-derived NK cell cytotoxicity by dendritic cells. J Immunol 2001;166:1590–1600.
47. Udono H, Srivastava PK. Comparison of tumor-specific immunogenicities of stress-induced protein gp96, hsp90, and hsp70. J Immunol 1994;152:5398–5403.

Original Contribution

Complete Regression of Hereditary Melanoma in a Mouse Model by Repeated Hyperthermia Using Magnetite Cationic Liposomes

AKIRA ITO[1], YOKO NAKAHARA[1], MASATAKE FUJIOKA[1], TAKESHI KOBAYASHI[1+], KOZUE TAKEDA[2], IZUMI NAKASHIMA[2], HIROYUKI HONDA[1*]

[1]Department of Biotechnology, School of Engineering, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan

[2]Department of Immunology, Nagoya University Graduate School of Medicine, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

[+]Present address: School of Bioscience and Biotechnology, Chubu University, Matsumoto-cho 1200, Kasugai, Aichi 487-8501, Japan

Abstract: Magnetite cationic liposomes (MCLs) have a positive surface charge, and have been used as heating mediators for intracellular hyperthermia because they generate heat in an alternating magnetic field (AMF) due to hysteresis loss. In our previous paper, hyperthermia using MCLs was applied to animals having several types of tumor, and a strong antitumor effect was observed in those animal models using transplantable tumor cell lines. In the present study, our protocol was applied to a hereditary melanoma model; primary skin melanoma developing in a metallothionein (MT)-I/*ret* transgenic mouse line. MCLs were injected into a melanoma nodule (size, 5-7 mm) in MT/*ret* transgenic mice, which were subjected to AMF for 30 min. The temperature at the surface of the tumor reached 45°C and was maintained by adjusting the magnetic field intensity. Hyperthermia treatment was repeated three times at 24 h intervals (repeated hyperthermia; RH), and RH was carried out until complete tumor regression was observed. Complete tumor regression was achieved in all mice treated once, twice or three times with RH. Furthermore, tumors successfully treated by RH did not undergo regrowth for 120 d post-treatment, and significant elongation of survival was observed. These results suggest that MCL-mediated hyperthermia is a potent approach to treat malignant melanoma.

Key words: hyperthermia, liposome, magnetite, melanoma, metallothionein-I/*ret* transgenic mouse

Introduction

Hyperthermia has been used for many years to treat a wide variety of tumors in both experimental animals and patients[1]. In Japan, the most commonly used heating method in clinical settings is

Received 8 Febrary 2005, Accepted 28 March 2005. *Corresponding author, Tel: +81-52-789-3215; Fax: +81-52-789-3214; E-mail: honda@nubio.nagoya-u.ac.jp

capacitive heating using a radiofrequency (RF) electric field[2]. However, specifically heating tumors by capacitive heating using an RF electric field is difficult because the heating characteristics are influenced by various factors, such as tumor size, position of electrodes, and adhesion of electrodes at uneven sites. Hyperthermia produced by heating mediators is a promising approach for specifically heating tumors without damaging normal tissues[3)4)]. Previously, studies have examined inductive heating methods in which the heating mediators are sub-micron magnetic particles[5-7)]. We have developed magnetite cationic liposomes (MCLs) as mediators of intracellular hyperthermia[8-10)]. These cationic liposomes exhibit improved adsorption and accumulation in tumor cells, and have 10-fold higher affinity for tumor cells than neutrally charged magnetoliposomes[8)], thus suggesting that MCLs are superior mediators of hyperthermia. We previously demonstrated the efficacy of MCL-mediated hyperthermia in animals with several cell lines, including B16 mouse melanoma[11)], T-9 rat glioma[9)], Os515 hamster osteosarcoma[12)], MM46 mouse mammary carcinoma[13)], PLS 10 rat prostate cancer[14)], and VX-7 squamous cell carcinoma in rabbit tongue[15)]. Although MCL-mediated hyperthermia was found to be very effective for inducing complete tumor regression in transplantable tumor models, no studies have used a hereditary cancer model, which is thought to be good approximation of cancer patients.

To date, various types of transgenic mouse have been produced to investigate the function of oncogenes in the process of cell differentiation of cells *in vivo*[16)17)]. The *ret* oncogene was activated by DNA rearrangement of the *ret* proto-oncogene with other cellular sequences during NIH3T3 transfection assay[18)19)]. The *ret* proto-oncogene encodes a receptor-type tyrosine kinase and is often expressed in human cell lines or tumors of neuroectodermal origin, such as neuroblastoma, pheochromocytoma and thyroid medullary carcinoma[20-23)]. Previously, in order to further investigate the action of the *ret* protein in various tissues, the mouse metallothionein (MT)-I promoter, which is known to function in almost all tissues[24)], was used. Surprisingly, four independent transgenic lines showed hyperpigmented skin due to aberrant melanogenesis[25)], and the melanocytic tumor that developed in MT/*ret* transgenic mice of line 304 finally progressed to melanoma accompanied by distant metastasis[26)27)]. In this transgenic mouse line, tumors developed mainly in the skin.

An effective protocol for melanoma therapy is urgently needed because of the recent reduction in the Earth's ozone layer, which blocks the sun's ultraviolet rays[28)] and the increasing incidence of melanoma, which is occurring at a greater rate than that of any other cancer[29)]. In addition, a relatively high percentage of melanoma is hereditary (6-14 %)[30)31)]. In the present study, we assessed the feasibility and potential of MCL-mediated hyperthermia to treat hereditary melanoma in a MT/*ret* transgenic mouse line.

Materials and Methods

Preparation of MCLs

Magnetite particles were kindly donated by Toda Kogyo Co. (Hiroshima ; average particle size, 10 nm). MCLs were prepared using a previously described sonication method, with slight modification[8)]. Briefly, 1 ml of colloidal magnetite was coated with a lipid membrane consisting of *N*- (α-trimethylammonioacetyl) didodecyl-*D*-glutamate chloride (Sogo Pharmaceutical Co., Tokyo), dilauroylphosphatidylcholine and dioleoylphosphatidylethanolamine (Sigma Chemical Co., St. Louis,

MO) at a molar ratio of 1 : 2 : 2. Magnetite concentration was measured using the potassium thiocyanate method[32]. In the present paper, MCL concentration is expressed as net magnetite concentration. Concentration of MCLs was adjusted to 20 mg/ml using phosphate-buffered saline (PBS).

MCL-mediated hyperthermia in MT/ret transgenic mice

MT/*ret* transgenic mice had no tumors at birth. Thereafter, multiple tumors with the histologic appearance of typical melanoma[26] developed in the skin throughout the body, and tumor size reached 5-7 mm in diameter at an average of 5.4 months after birth. MT/*ret* transgenic mice with tumor sizes of 5-7 mm were randomly divided into two groups ; a control group, which received no treatment, and hyperthermia group, which received MCL-mediated hyperthermia.

For the hyperthermia group, tumor-bearing mice were anesthetized with pentobarbital sodium (50 mg/ kg i. p.). Under anesthesia, a syringe (26 G needle) containing MCLs was inserted longitudinally into each tumor subcutaneously from the tumor edge. MCL solution (0.2 ml, 20 mg-magnetite/ml) was injected using an infusion pump (SP100i ; World Precision Instruments, Sarasota, FL, USA) for 30 min. After injection of MCLs, mice were subjected to an alternating magnetic field (AMF) for 30 min. AMF was generated by a horizontal coil (inner diameter : 7 cm ; length : 7 cm) with a transistor inverter (LTG-100-05 ; Dai-ichi High Frequency, Tokyo). The magnetic field frequency was 118 kHz. The mouse was placed inside the coil such that the tumor was positioned at the center. Temperatures in the rectum and at the surface of the tumor during AMF irradiation were measured with an optical fiber probe (FX-9020 ; Anritsu Meter, Tokyo). Hyperthermia treatment was repeated three times at 24 h intervals (repeated hyperthermia ; RH), and RH was carried out until complete tumor regression was achieved ; if partial tumor regrowth occurred after RH, a further 0.2 ml of MCL solution was injected into the tumor and RH was again conducted. Tumor diameter was measured every 3 days, and size was determined by the following formula ; Tumor size $=0.5 \times$ (length + width), where length and width are measured in millimeters.

Statistical analysis

For survival analysis, differences in survival rates were analyzed by log-rank test in WinSTAT (Light Stone International, Tokyo). A P value of < 0.05 was considered to indicate statistical significance.

Animal experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared under the direction of the Office of the Prime Minister of Japan.

Results

The temperature increase during hyperthermia using MCLs was investigated. After melanoma nodules had grown to 5-7 mm in diameter, 0.2 ml of MCL solution (net magnetite weight : 4 mg) was injected into the center of tumors, and mice were subjected to AMF for 30 min. Fig. 1 shows the temperature profile at the surface of tumors during AMF irradiation. Tumor temperature increased rapidly, reaching 45°C within 5 min, and was maintained at 45°C by adjusting the magnetic field intensity.