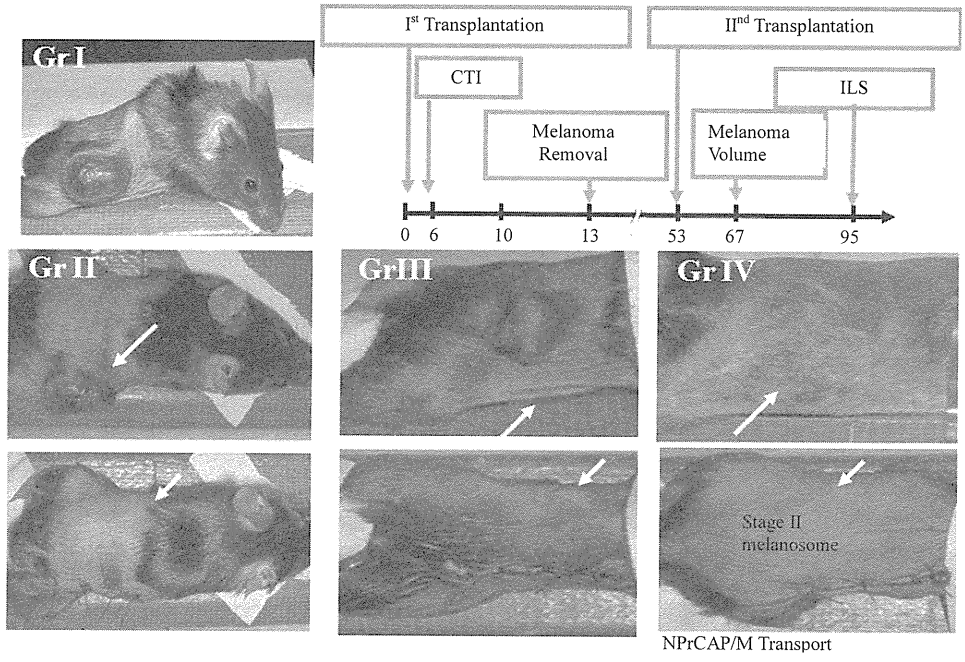
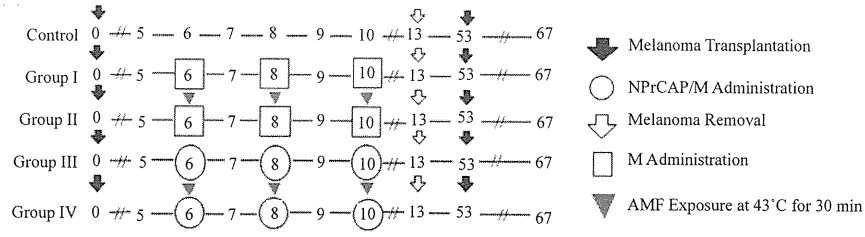


Figure 7. Growth inhibition of melanoma cells by primary first transplant and secondary re-challenge transplant in C57 black mice. Gr.I; control without NPrCAP/M treatment and second re-challnege melanoma transplant. Gr.II: control without NPrCAP/M treatment, but received second re-challenge of melanoma; Gr.III: control with NPrCAP/M treatment without AMF exposure, but received second re-challenge melanoma transplant. Gr.IV: mice with NPrCAP/M and AMF exposure as well as second re- challenge melanoma transplant. After removal of the first melanoma transplant, all mice (except Gr.I) received the second re-challenge melanoma transplant on the opposite site of trunk.



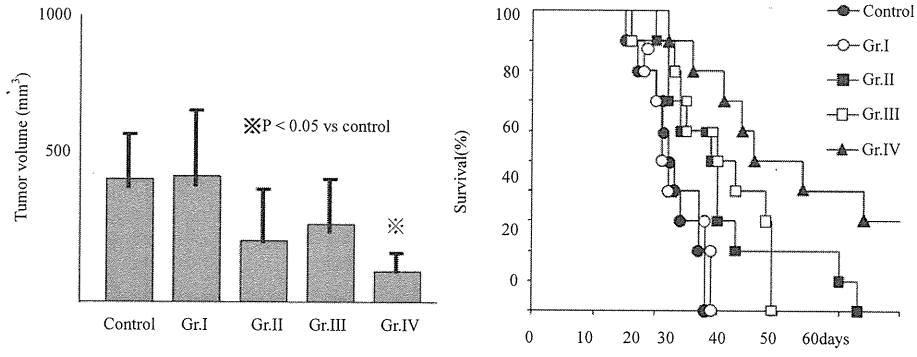


Figure 8. Melanoma growth and survival of melanoma- bearing mice by CTI therapy using NPrCAP/M with and without AMF exposure. (a) Protocols of Group I, II, III and IV of experimental mice; (b) Tumor volumes of re-challenge B16F1 melanoma on day 14; (c) Kaplan-Meier survival after tumor re-challenge.

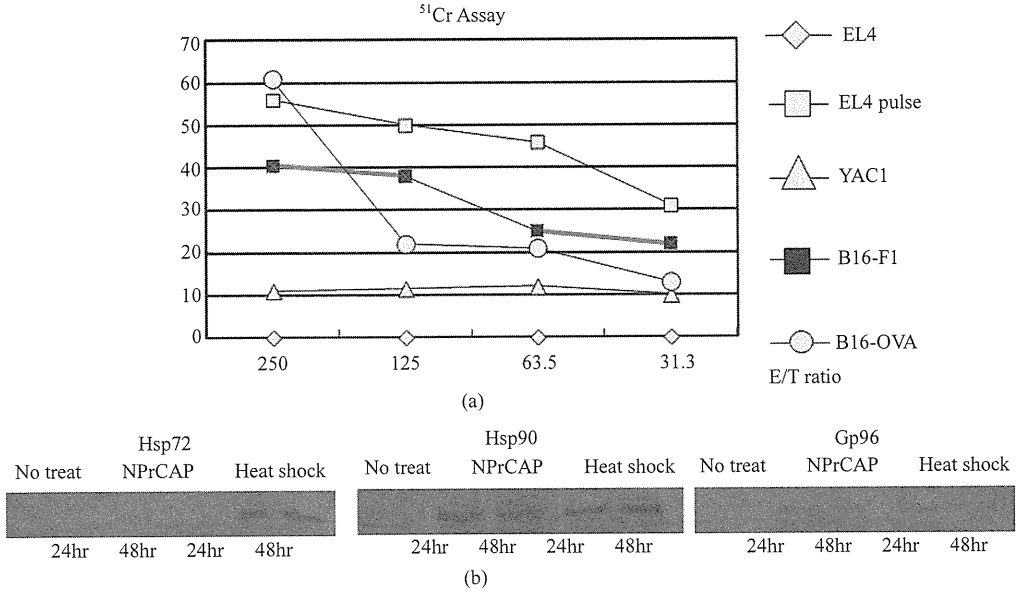


Figure 9. Hyperthermia of melanoma cells using B16-OVA cells for induction of CTL (a) and generation of HSPs (b) in CTI therapy. (a) Cytotoxic activity of spleen cells after CTI therapy against B16-OVA cells, B16F1 cells, EL4 cells, EL4 cells pulsed with SL8 peptide or YAC-1 cells was determin- ed by standard 51Cr-release assay. B16-OVA cells were subjected to hyperthermia using NPrCAP/M with AMF exposure in vitro. (b)The expression of Hsp72, Hsp90 and Gp96 was determined by western blotting with an anti- Hsp72 mAb, anti-Hsp90 mAb or anti KDEL mAb.

3.3. Production of Heat Shock Protein by Chemothermo-Immunotherapy Using NPrCAP/ Magnetite Nanoparticle Conjugates

It has been reported that the intracellular hyperthermia using magnetic nanoparticles is effective for treating certain types of cancer in not only primary but also metastatic lesions [37-43]. Incorporated magnetic nanoparticles generate heat within the cells after exposure to the AMF due to hysteresis loss or relaxational loss [44,45]. Hyperthermic treatment using CMLs, which are cationic liposomes containing 10 nm magnetite nanoparticles, induced antitumor immunity by enhancement of HSP expression [38,46-48].

In our animal study, those animals bearing B16F1 and B16F10 melanoma cells showed, to certain degree, rejecttion of second re-challenge melanoma transplantation by administration of both NPrCAP alone and NPrCAP/M minus AMF exposure [49]. Our working hypothesis for this finding is that there is a difference in the cytotoxic mechanism and immunogenic property of NPrCAP/M between experimental groups with and without AMF exposure. The animals with NPrCAP/M without AMF exposure resulted in non-necrotic, apoptotic cell death. The animals with NPrCAP/M plus AMF exposure, on the other hand, resulted in non-apoptotic, necrotic cell death with immune complex production of melanoma peptide as well as Hsp70 and a small amount of Hsp 90. The latter group of NPrCAP/M plus AMF exposure showed the most significant growth inhibition of the re-challenged melanoma growth which resulted in the almost complete survival of the host animals as long as for 3 months that we have conducted our experimental protocol [34]. In the latter study [34], we also found that repeated hyperthermia (3 cycles of NPrCAP/M administration and AMF irradiation) was required to induce the maximal antitumor immune response.

In the study using B16-OVA cells, the hyperthermia of melanoma cells using NPrCAP/M with AMF exposure further showed antitumor immune responses via cross-presentation of HSP-chaperoned antigen (**Figures 9(a), (b)**) (**Figures 10(a)-(d)**). Moreover, the HSPs-antigen peptide complex released from melanoma cells treated with this intracellular hyperthermia was taken-up by dendritic cells (DCs) and cross-presented HSP-chaperoned peptide in the context of MHC class I molecules [49]. As stated above, our CTI therapy with AMF exposure induced NPrCAP- as well as heat-mediated melanoma cell necrosis to NPrCAP/M incorporated cells. If melanoma cells escaped from necrotic cell death, repeated hyperthermia should produce necrotic cell death of previously heat shocked-melanoma cells in which HSPs were induced. In addition, our data of CTI therapy with AMF exposure using B16-OVA cells suggested that Hsp72/Hsc73, Hsp90, and ER-resident HSPs participated

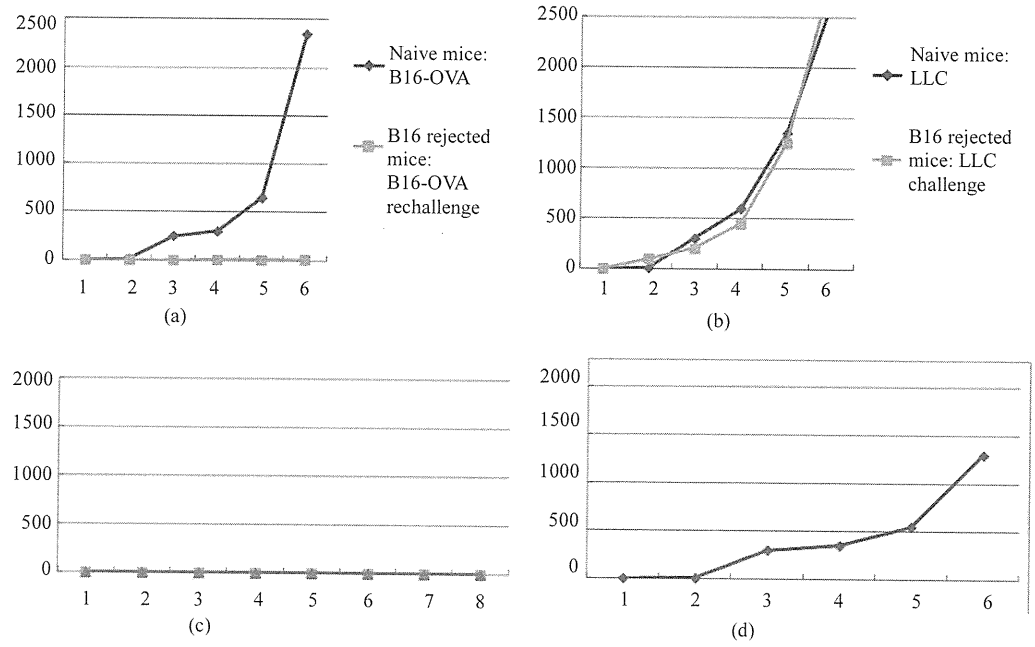


Figure 10. Tumor-specific immunity induced by CTI therapy. Naïve mice or mice cured by CTI therapy were re- challenged with B16-OVA melanoma cells (a) or lung carcinoma LLC (b). Failure of the melanoma growth by secondary re-challenge after CTI therapy to the primary melanoma transplant (a). There was no growth inhibition of LLC cells in mice which received transplantation of B16-OVA melanoma cells and received CTI therapy (b). Antitumor immunity is dependent on $CD8^+$ T cells. Mice cured by CTI therapy were depleted of $CD4^+$ or $CD8^+$ T cells by an intraperitoneal injection of rat IgG (c) or anti-CD4 mAb (c) or anti-CD8 mAb (d). The growth inhibition of secondary re-challenge melanoma transplant failed after pretreatment with anti-CD8 mAb, but not by anti-CD4 mAb.

in the induction of $CD8^+$ T cell response. In particular, among HSPs, Hsp72 was largely responsible for the augmented antigen presentation to $CD8^+$ T cells. As Hsp72 is known to up-regulate in response to hyperthermia or heat shock treatment [46], newly synthesized Hsp72 has a chance to bind to the heat-denatured melanoma-associated antigen.

3.4. Melanocytotoxic and Immunogenic Properties of NPrCAP Compared to Hydroquinone

Monobenzyl ether of hydroquinone has long been known to produce the skin depigmentation at both drug-applied area by direct chemical reaction with tyrosinase and non-applied distant area by immune reaction with still unknown mechanism [50-53]. The melanogenesis-related cytotoxicity primarily derives from tyrosinase-mediated formation of dopaquinone and other quinone intermediates, which produce reactive oxygen species such as superoxide and H_2O_2 [19,54-56]. This unique biological property of melanin intermediates not only causes cell death, but also may produce immunogenic properties. We postulated that the cytotoxic action of NAcCAP and NPrCAP appears to involve two major biological processes. One is cytostatic process which derives from the DNA synthesis inhibition through the interaction of quinone and free radicals with SH-enzymes and thymidine synthase. Another is the cytocidal process by damage of DNA and mitochondrial ATP through oxidative stress and interaction with SH-enzyme [21]. They bind protein disulphide isomerise [57]. Although we have not yet studied which one of these two processes is responsible for the immune reaction, it is likely that the cytocidal process of NPrCAP is involved in the induction of immune reaction.

Monobenzy ether of hydroquinone was shown to produce a reactive ortho-quinone generated by tyrosinase-catalyzed oxidation and self-coupling and thiol conjugation reactions [58]. It was also shown to induce cell death without activating the caspase cascade or DNA fragmentation, indicating that the death pathway is non- apoptotic [58,59]. It was further reported that mono- benzyl ether of hydroquinone induced the immunogenicity to melanocytes and melanoma cells by forming quinonehaptens to tyrosinase protein and by inducing the release of tyrosinase- and melanoma antigen recognized by T cells-1 (MART-1) containing $CD63^+$ exosomes following melanosome oxidative stress induction. The drug further augmented the processing and shedding of melanocyte

differentiation antigens by inducing melanosome autophagy and enhanced tyrosinase ubiquitination, ultimately activating DCs, which induced cytotoxic human melanoma-reactive cells. These T cells eradicated melanoma *in vivo* [59]. It is necessary to examine if NPrCAP will also take an immune-biological process similar to that reported in the case of mono- benzyl ether of hydroquinone.

4. Discussion

The immune system can respond to cancer cells in two ways: by reacting against tumor-specific antigens (molecules that are unique to cancer cells) or against tumor-associated antigens (molecules that are expressed differentially by cancer cells and normal cells) [60]. The immunotherapy for cancer cells is further subdivided into immunotherapy with antibodies and T cells, therapeutic cancer vaccines, therapeutic vaccines combined with chemotherapy and immunoprevention of cancer cells. Several clinical trials using melanoma peptides or an antibody that blocks cytotoxic T-lymphocyte-associated antigen on lymphocytes have been shown to improve overall melanoma survival [61-63]. Exploitation of a specific biological property to cancer cells may, however, be another approach for developing novel cancer-targeted drugs. Promising oncogene-targeted melanoma therapy has successfully introduced recently [64].

Hyperthermia increases the expression of intracellular HSPs which is important in and necessary for the induction of antitumor immunity [46,65]. Over expression of HSPs, such as Hsp 70, increases tumor immunogenicity by augmenting the chaperoning ability of antigenic peptides and presentation of antigenic peptides in MHC class I molecules [66,67]. In this process professional antigen presenting DCs play unique and important roles in taking up, processing and presenting exogenous antigens in association with MHC class I molecules. Our working hypothesis for induction of *in situ* vaccination immunotherapy is that CTI therapy causes degradation of melanoma tissues which results in the release of HSP/melanoma antigen complex. This complex is taken up by professional antigen-presenting DCs through HSP receptor (**Figure 11**).

Based upon these animal experiments, a preliminary human clinical trial has been carried out by employing NPrCAP/PEG/M plus AMF after we obtained the approval of our human clinical trial for a limited number of stage III and IV melanoma patients (Clinical Trial Research No. 18-67, Sapporo Medical University). The therapeutic protocol followed the basically identical experimental schedule as that of animal experiments. In the clinical trials, however, we utilized NPrCAP/PEG/M which was made by conjugating polyethylene glycol

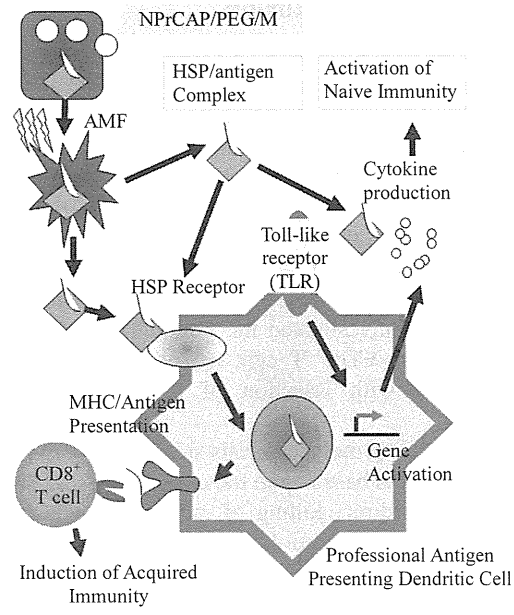


Figure 11. Scheme of intracellular hyperthermia using NPrCAP/PEG/M with AMF exposure. NPrCAP/PEG/M nanoparticles are selectively incorporated into melanoma cells. Intracellular hyperthermia can induce necrotic cell death and adjacent live melanoma cells suffer heat shock, resulting in increased level of intracellular HSP-peptide complexes. Repeated hyperthermia turns heat-shocked cells to necrotic cells, leading to the release of HSPs-peptide complexes into extracellular milieu. The released HSPs-peptide complexes are taken-up by DCs. Then, DCs migrate into regional lymph nodes and cross-present HSP chaperoned antigenic peptides to $CD8^+$ T cells in the context of MHC class I molecules, thereby inducing anti-melanoma cytotoxic $CD8^+$ T cells.

between NPrCAP and magnetite nanoparticles. Among two of four patients showed complete and partial responses to our treatment and have been able to carry out normal daily activities after CTI therapy. In one patient, for example, four distant cutaneous metastasis sites were evaluated and either significant regression or shrinkage of all of these four melanoma lesions was seen. The patient was able to survive 30 months after several trials of CTI therapy. The pathological and immunological specimens revealed dense aggregation of lymphocytes and macrophages at the site of CTI therapy. Importantly there was a trend to have an almost identical distribution of $CD8^+$ T cells and MHC class 1 positive cells. Another patient had many lymph node metastases, but still has been surviving more than 32 months. In order to evaluate the overall therapeutic effect to advanced melanoma, it is important to have larger-scaled clinical trials and define concisely the molecular interaction between chemotherapeutic and thermo-immunotherapeutic effect in our CTI therapy.

5. Summary and Conclusion

In this study, we examined to what extent the conjugates of magnetite nanoparticles and melanogenesis substrate can generate melanoma-targeted vaccines through the chemotherapeutic and thermotherapeutic effects on primary transplant of B16 mouse melanoma cells with and without AMF exposure (heat generation). Specifically we evaluated the immunotherapeutic effect on the second, re-challenge transplant of the same melanoma cells to see if the growth of distant metastatic melanomas can be inhibited. We also investigated the possible association of HSP production, $CD8^+$ T cell activation and MHC expression along with rejection of the re-challenge melanoma.

Our approach using melanogenesis substrate and magnetite nanoparticles is based upon the expectation that the combination of 1) direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effect of melanogenesis-targeted drug (NPrCAP/M) and 2) indirect killing by immune reaction (*in situ* peptide vaccine) after exposure to AMF. It is hoped from these rationales that a tumor-specific DDS is developed by NPrCAP and selective cell death can be achieved by exposure of conjugates of NPrCAP/M nanoparticles to AMF, which then can induce HSP expression through either necrotic or non-necrotic process or combination of the two, without damaging non-cancerous tissues. Finally a novel immunotherapy targeted to metastatic melanoma lesions is achieved through "*in situ* peptide vaccine".

6. Acknowledgements

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

We also express our sincere appreciation to Drs. Akiko Sato, Makito Sato, Yasue Ishii-Osai and Tomoaki Takada who devoted to carry out basic experiments and clinical trials of this study.

REFERENCES

[1] C. M. Balch, A. C. Buzaid, S. J. Soong, M. B. Atkins, N. Cascinelli, D. G. Coit, I. D. Fleming, J. E. Gershenwald, A. Houghton Jr., J. M. Kirkwood, K. M. McMasters, M. F. Mihm, D. L. Morton, D. S. Reintgen, M. I. Ross, A. Sober, J. A. Thompson and J. F. Thompson, "Final Version of the American Joint Committee on Cancer Staging System for Cutaneous Melanoma," *Journal of Clinical Oncology*, Vol. 19, No. 16, 2001, pp. 3635-3648.

[2] A. Ito, M. Shinkai, H. Honda and T. Kobayashi, "Medical Application of Functionalized Magnetic Nanoparticles," *Journal of Bioscience and Bioengineering*, Vol. 100, No. 1, 2005, pp. 1-11. doi:10.1263/jbb.100.1

[3] N. Kawai, A. Ito, Y. Nakahara, M. Futakuchi, T. Shirai, H. Honda, T. Kobayashi and K. Kohri, "Anticancer Effect of Hyperthermia on Prostate Cancer Mediated by Magnetite Cationic Liposomes and Immune-Response Induction in Transplanted Syngeneic Rats," *The Prostate*, Vol. 64, No. 4, 2005, pp. 373-381. doi:10.1002/pros.20253

[4] M. Yanase, M. Shinkai, H. Honda, T. Wakabayashi and J. Yoshida, T Kobayashi, "Antitumor Immunity Induction by Intracellular Hyperthermia Using Magnetite Cationic Liposomes," *Cancer Science*, Vol. 89, No. 7, 1998, pp. 775-782. doi:10.1111/j.1349-7006.1998.tb03283.x

[5] M. Shinkai, M. Yanase, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Intracellular Hyperthermia for Cancer Using Magnetite Cationic Liposomes: *In vitro* Study," *Cancer Science*, Vol. 87, No. 11, 1996, pp. 1179-1183. doi:10.1111/j.1349-7006.1996.tb03129.x

[6] A. Ménoret and R. Chandawarkar, "Heat-Shock Protein-Based Anticancer Immunotherapy: An Idea Whose Time Has Come," *Seminars in Oncology*, Vol. 25, No. 6, 1998, pp. 654-660.

[7] P. K. Srivastava, A. Ménoret, S. Basu, R. Binder and K. Quade, "Heat Shock Proteins Come of Age: Primitive Functions Acquired New Roles in an Adaptive World," *Immunity*, Vol. 8, No. 6, 1998, pp. 657-665. doi:10.1016/S1074-7613(00)80570-1

[8] Y. Tamura, N. Tsuboi, N. Sato and K. Kikuchi, "70 kDa Heat Shock Cognate Protein Is a Transformation-Associated Antigen and a Possible Target for the Host's Anti-Tumor Immunity," *The Journal of Immunology*, Vol. 151, No. 10, 1993, pp. 5516-5524.

[9] Y. Tamura, P. Peng, K. Liu, M. Daou and P. K. Srivastava, "Immunotherapy of Tumors with Autologous Tumor-Derived Heat Shock Protein Preparations," *Science*, Vol. 278, No. 5335, 1997, pp. 117-120. doi:10.1126/science.278.5335.117

[10] Y. Tamura and N. Sato, "Heat Shock Proteins: Chaperoning of Innate and Adaptive Immunities," *Japanese Journal of Hyperthermic Oncology*, Vol. 19, No. 3, 2003, pp. 131-139.

[11] P. K. Srivastava, "Immunotherapy for Human Cancer Using Heat Shock Protein-Peptide Complexes," *Current Oncology Reports*, Vol. 7, No. 2, 2005, pp. 104-108. doi:10.1007/s11912-005-0035-8

[12] Y. Tamura, S. Takashima, J.M. Cho, W. Qi, K. Kamiguchi, T. Torigoe, S. Takahashi, I. Hirai, N. Sato and K. Kikuchi, "Inhibition of Natural Killer Cell Cytotoxicity by Cell Growth-Related Molecules," *Cancer Science*, Vol. 87, No. 6, 1996, pp. 623-630. doi:10.1111/j.1349-7006.1996.tb00269.x

[13] G. Ueda, Y. Tamura, I. Hirai, K. Kamiguchi, S. Ichimiya, T. Torigoe, H. Hiratsuka, H. Sunakawa and N. Sato, "Tumor-Derived Heat Shock Protein 70-Pulsed Dendritic Cells Elicit Tumor-Specific Cytotoxic T Lymphocytes (Ctls) and Tumor Immunity," *Cancer Science*, Vol. 95, No. 1, 2004, pp. 248-253. doi:10.1111/j.1349-7006.2004.tb02211.x

[14] A. Ito, H. Honda and T. Kobayashi, "Cancer Immuno-

therapy Based on Intracellular Hyperthermia Using Magnetite Nanoparticles: A Novel Concept of 'Heat-Con-Trolled Necrosis' with Heat Shock Protein Expression," *Cancer Immunology, Immunotherapy*, Vol. 55, No. 3, 2006, pp. 320-328. doi:10.1007/s00262-005-0049-y

[15] H. Shi, T. Cao, J. E. Connolly, L. Monnet, L. Bennett, S. Chapel, C. Bagnis, P. Mannoni, J. Davoust, A. K. Palucka and J. Banchereau, "Hyperthermia Enhances CTL Cross-Priming," *The Journal of Immunology*, Vol. 176, No. 4, 2006, pp. 2134-2141.

[16] K. Jimbow, T. Iwashina, F. Alena, K. Yamada, J. Pankovich and T. Umemura, "Exploitation of Pigment Biosynthesis Pathway as a Selective Chemotherapeutic Approach for Malignant Melanoma," *Journal of Investigative Dermatology*, Vol. 100, No. S2, 1993, pp. S231-S238.

[17] F. Alena, T. Ishikawa, A. Gili and K. Jimbow, "Selective *in Vivo* Accumulation of N-acetyl-4-S-Cysteaminylphenol in B16F10 Murine Melanoma and Enhancement of Its *in Vitro* and *in Vivo* Antimelanoma Effect by Combination of Buthionine Sulfoximine," *Cancer Research*, Vol. 54, No. 10, 1994, pp. 2661-2666.

[18] J. M. Pankovich and K. Jimbow, "Tyrosine Transport in a Human Melanoma Cell Line as a Basis for Selective Transport of Cytotoxic Analogues," *Biochemical Journal*, Vol. 280, 1991, pp. 721-725.

[19] K. Reszka and K. Jimbow, "Electron Donor and Acceptor Properties of Melanin Pigments in the Skin," In: J. Fuchs and L. Packer, *Oxidative Stress in Dermatology*, Marcel Dekker Inc., New York, 1993. pp. 287-320.

[20] M. Tandon, P. D. Thomas, M. Shokravi, S. Singh, S. Samra, D. Chang and K. Jimbow, "Synthesis and Antitumour Effect of the Melanogenesis-Based Anti-Melanoma Agent N-Propionyl-4-S-Cysteaminylphenol," *Biochemical Pharmacology*, Vol. 15, No. 12, 1998, pp. 2023-2029. doi:10.1016/S0006-2952(98)00090-2

[21] P. D. Thomas, H. Kishi, H. Cao, M. Ota, T. Yamashita, S. Singh and K. Jimbow, "Selective Incorporation and Specific Cytocidal Effect as the Cellular Basis for the Antimelanoma Action of Sulphur Containing Tyrosine Analogs," *Journal of Investigative Dermatology*, Vol. 113, 1999, pp. 928-934. doi:10.1046/j.1523-1747.1999.00781.x

[22] J. Dakour, M. Vinayagamoorthy, H. Chen, D. Luo, W. Dixon and K. Jimbow, "Identification of A cDNA for Ca^{2+}-Binding Calnexin-Like Phosphoprotein (p90) on Melanosomes in Normal and Malignant Human Melanocytes," *Experimental Cell Research*, Vol. 209, No. 2, 1993, pp. 288-300. doi:10.1006/excr.1993.1313

[23] K. Toyofuku, I. Wada, K. Hirosaki, J. S. Park, Y. Hori and K. Jimbow, "Involvement of Calnexin in Maturation of Tyrosinase as a Molecular Chaperone," *Journal of Biochemistry*, Vol. 125, 1999, pp. 82-89.

[24] K. Jimbow, P.F. Gomez, K. Toyofuku, D. Chang, S. Miura, H. Tsujiya and J.S. Park, "Biological Role of Tyrosinase Related Protein and Its Biosynthesis and Transport from TGN to Stage I Melanosome, Late Endosome, through Gene Transfection Study," *Pigment Cell Research*, Vol. 10, No. 4, 1997, pp. 206-213. doi:10.1111/j.1600-0749.1997.tb00486.x

[25] K. Jimbow, J. S. Park, F. Kato, K. Hirosaki, K. Toyofuku, C. Hua and T. Yamashita, "Assembly, Target Signal and Intracellular Transport of Tyrosinase Gene Family Protein in the Initial Stage of Melanosome Biogenesis," *Pigment Cell Research*, Vol. 13, No. 4, 2000, pp. 222-229. doi:10.1034/j.1600-0749.2000.130403.x

[26] K. Jimbow, C. Hua, P. F. Gomez, K. Hirosaki, K. Shinoda, T. G. Salopek, H. Matsusaka, H. Y. Jin and T. Yamashita, "Intracellular Vesicular Trafficking of Tyrosinase Gene Family Protein in Eu- and Pheomelanosome Biogenesis," *Pigment Cell Research*, Vol. 13, No. S8, 2000, pp. S110-S117. doi:10.1034/j.1600-0749.13.s8.20.x

[27] T. Miura, K. Jimbow and S. Ito, "The *in Vivo* Antimelanoma Effect of 4-S-Cysteaminylphenol and Its N-Acetyl Derivative," *International Journal of Cancer*, Vol. 46, No. 5, 1990, pp. 931-934. doi:10.1002/ijc.2910460530

[28] M. Tandon, P. D. Thomas, M. Shokravi, S. Singh, S. Samra, D. Chang and K. Jimbow, "Synthesis and Antitumour Effect of the Melanogenesis-Based Antimelanoma Agent N-Propionyl-4-S-Cysteaminylphenol," *Biochemical Pharmacology*, Vol. 55, No. 12, 1998, pp. 2023-2029. doi:10.1016/S0006-2952(98)00090-2

[29] S. Ito, T. Kato, K. Ishikawa, T. Kasuga and K. Jimbow, "Mechanism of Selective Toxicity of 4-S-Cysteinylphenol and 4-S-Cysteaminylphenol to Melanocytes," *Biochemical Pharmacology*, Vol. 36, No. 12, 1987, pp. 2007-2011. doi:10.1016/0006-2952(87)90501-6

[30] A. Gili, P. D. Thomas, M. Ota and K. Jimbow, "Comparison of *in Vitro* Cytotoxicity of N-Acetyl and N-Propionyl Derivatives of Phenolic Thioether Amines in Melanoma and Neuroblastoma Cells and the Relationship to Tyrosinase and Tyrosine Hydroxylase Enzyme Activity," *Melanoma Research*, Vol. 10, No. 1, 2000, pp. 9-15.

[31] B. Thiesen and A. Jordan, "Clinical Applications of Magnetic Nanoparticles for Hyperthermia," *International Jour- nal of Hyperthermia*, Vol. 24, No. 6, 2008, pp. 467-474.

[32] M. Johannsen, U. Gneveckow, L. Eckelt, A. Feussner, N. Waldöfner, R. Scholz, S. Deger, P. Wust, S. A. Loening and A. Jordan, "Clinical Hyperthermia of Prostate Cancer Using Magnetic Nanoparticles: Presentation of a New Interstitial Technique," *International Journal of Hyperthermia*, Vol. 21, No. 7, 2005, pp. 637-647.

[33] A. Ito, M. Fujioka, T. Yoshida, K. Wakamatsu, S. Ito, T. Yamashita, K. Jimbow and H. Honda, "4-S-Cysteaminylphenol-Loaded Magnetite Cationic Liposomes for Combination Therapy of Hyperthermia with Chemotherapy against Malignant Melanoma," *Cancer Science*, Vol. 98, No. 3, 2007, pp. 424-430. doi:10.1111/j.1349-7006.2006.00382.x

[34] T. Takada, T. Yamashita, M. Sato, A. Sato, I. Ono, Y. Tamura, N. Sato, A. Miyamoto, A. Ito, H. Honda, K. Wakamatsu, S. Ito and K. Jimbow, "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," *Journal of Biomedicine and Biotechnology*, 2009, Article ID: 457936. doi:10.1155/2009/457936

[35] M. Sato, T. Yamashita, M. Ohkura, Y. Osai, A. Sato, T. Takada, H. Matsusaka, I. Ono, Y. Tamura, N. Sato, Y. Sasaki, A. Ito, H. Honda, K. Wakamatsu, S. Ito and K. Jimbow, "N-Propionyl-Cysteaminyl-Phenol-Magnetite Conjugate (NPrCAP/M) Is a Nanoparticle for the Targeted Growth Suppression of Melanoma Cells," *Journal of Investigative Dermatology*, Vol. 129, 2009, pp. 2233-2241. doi:10.1038/jid.2009.39

[36] M. Suzuki, M. Shinkai, H. Honda and T. Kobayashi, "Anticancer Effect and Immune Induction by Hyperthermia of Malignant Melanoma Using Magnetite Cationic Liposomes," *Melanoma Research*, Vol. 13, No. 2, 2003, pp. 129-135. doi:10.1097/00008390-200304000-00004

[37] M. Yanase, M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Intracellular Hyperthermia for Cancer Using Magnetite Cationic Liposomes: An *in Vivo* Study," *Cancer Science*, Vol. 89, No. 4, 1998, pp. 463-469. doi:10.1111/j.1349-7006.1998.tb00586.x

[38] N. Kawai, A. Ito, Y. Nakahara, M. Futakuchi, T. Shirai, H. Honda, T. Kobayashi and K. Kohri, "Anticancer Effect of Hyperthermia on Prostate Cancer Mediated by Magnetite Cationic Liposomes and Immunere-Sponse Induction in Transplanted Syngeneic Rats," *Prostate*, Vol. 64, No. 4, 2005, pp. 373-381. doi:10.1002/pros.20253

[39] A. Ito, M. Shinkai, H. Honda and T. Kobayashi, "Medical Application off Unctionalized Magnetic Nanoparticles," *Journal of Bioscience and Bioengineering*, Vol. 100, No. 1, 2005, pp. 1-11. doi:10.1263/jbb.100.1

[40] M. Yanase, M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Antitumor Immunity Induction by Intracellular Hyperthermia Using Magnetite Cationic Liposomes," *Cancer Science*, Vol. 89, No. 7, 1998, pp. 775-782. doi:10.1111/j.1349-7006.1998.tb03283.x

[41] A. Ito, M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Augmentation of MHC Class I Antigen Presentation via Heat Shock Protein Expression by Hyperthermia," *Cancer Immunology, Immunotherapy*, Vol. 50, No. 10, 2001, pp. 515-522. doi:10.1007/s00262-001-0233-7

[42] A. Ito, T. Kobayashi and H. Honda, "A Mechanism of Antitumor Immunity Induced by Hyperthermia," *Japanese Journal of Hyperthermic Oncology*, Vol. 21, No. 1, 2005, pp. 1-19.

[43] A. Ito and K. Takeshi, "Intracellular Hyperthermia Using Magnetite Nanoparticles: A Novel Method for Hyperthermia Clinical Applications," *Thermal Medicine*, Vol. 24, No. 4, 2008, pp. 113-129. doi:10.3191/thermalmed.24.113

[44] M. Shinkai, M. Yanase, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Intracellular Hyperthermia for Cancer Using Magnetite Cationic Liposomes: *In Vitro* Study," *Cancer Science*, Vol. 87, No. 11, 1996, pp. 1179-1183. doi:10.1111/j.1349-7006.1996.tb03129.x

[45] R. Hergt, S. Dutz, R. Mueller and M. Zeisberger, "Magnetite Particle Hyperthermia: Nanoparticle Magnetism and Materials Development for Cancer Therapy," *Journal of Physics: Condensed Matter*, Vol. 18, No. 38, 2006, pp. S2919-S2934. doi:10.1088/0953-8984/18/38/S26

[46] A. Ito, M. Shinkai, H. Honda, K. Yoshikawa, S. Saga, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Heat Shock Protein 70 Expression Induces Antitumor Immunity during Intracellular Hyperthermia Using Magnetite Nanoparticles," *Cancer Immunology, Immunotherapy*, Vol. 52, No. 2, 2003, pp. 80-88.

[47] A. Ito, F. Matsuoka, H. Honda and T. Kobayashi, "Antitumor Effects of Combined Therapy of Recombinant Heat Shock Protein 70 and Hyperthermia Using Magnetic Nanoparticles in an Experimental Subcutaneous Murine Melanoma," *Cancer Immunology, Immunotherapy*, Vol. 53, No. 1, 2004, pp. 26-32. doi:10.1007/s00262-003-0416-5

[48] A. Ito, H. Honda and T. Kobayashi, "Cancer Immunotherapy Based on Intracellular Hyperthermia Using Magnetite Nanoparticles: A Novel Concept of 'Heat Controlled Necrosis' with Heat Shock Protein Expression," *Cancer Immunology, Immunotherapy*, Vol. 55, No. 3, 2006, pp. 320-328.doi:10.1007/s00262-005-0049-y

[49] P. Manini, A. Napolitano, W. Westerhof, P. A. Riley and M. d'Ischia, "A Reactive Ortho-Quinone Generated by Tyrosinase-Catalyzed Oxidation of the Skin Depigmenting Agent Monobenzone: Self-Coupling and Thiol-Conjugation Reactions and Possible Implications for Melanocyte Toxicity," *Chemical Research in Toxicology*, Vol. 22, No. 8, 2009, pp. 1398-1405. doi:10.1021/tx900018q

[50] A. Sato, Y. Tamura, N. Sato, T. Yamashita, T. Takada, M. Sato, Y. Osai, M. Okura, I. Ono, A. Ito, H. Honda, K. Wakamatsu, S. Ito and K. Jimbow, "Melanoma-Targeted Chemo-Thermo-Immuno (CTI)-Therapy Using N-Propionyl-4S-Cysteaminylphenol-Magnetite Nanoparticles Elicits CTL Response via Heat Shock Protein-Peptide Complex Release," *Cancer Science*, Vol. 101, No. , 2010, pp. 1939-1946. doi:10.1111/j.1349-7006.2010.01623.x

[51] K. Jimbow, H. Obata, M. A. Pathak and T. B. Fitzpatrick, "Mechanism of Depigmentation by Hydroquinone," *Journal of Investigative Dermatology*, Vol. 62, 1974, pp. 436-449. doi:10.1111/1523-1747.ep12701679

[52] J. J. Nordlund, B. Forget, J. Kirkwood and A. B. Lerner, "Dermatitis Produced by Applications of Monobenzone in Patients with Active Vitiligo," *Archives of Dermatology*, Vol. 121, No. 9, 1985, pp. 1141-1144. doi:10.1001/archderm.1985.01660090055013

[53] R. E. Boissy and P. Manga, "On the Etiology of Contact/Occupational Vitiligo," *Pigment Cell Research*, Vol. 17, No. 3, 2004, pp. 208-214. doi:10.1111/j.1600-0749.2004.00130.x

[54] C. J. Cooksey, K. Jimbow, E. J. Land and P. A. Riley, "Reactivity of Orthoquinones Involved in Tyrosi-Nase-Dependent Cytotoxicity: Differences between Alkylthio- and Alkoxy-Substituents," *Melanoma Research*, Vol. 2, No. 5-6, 1992, pp. 283-293. doi:10.1097/00008390-199212000-00001

[55] Y. Minamitsuji, K. Toyofuku, S. Sugiyama and K. Jimbow, "Sulphur Containing Tyrosinase Analogs Can Cause

Selective Melanocytoxicity Involving Tyrosinase-Mediated Apoptosis," *Journal of Investigative Dermatology*, Vol. 4, 1999, pp. S130-S136.

[56] K. Jimbow, Y. Miyake, K. Homma, K. Yasuda, Y. Izumi, A. Tsutsumi and S. Ito, "Characterization of Melanogenesis and Morphogenesis of Melanosomes by Physicochemical Properties of Melanin and Melanosomes in Malignant Melanoma," *Cancer Research*, Vol. 44, No. 3, 1984, pp. 1128-1134.

[57] P. G. Parsons, F. Favier, M. McEwan, T. Takahashi, K. Jimbow and S. Ito, "Action of Cysteaminylphenols on Human Melanoma Cells *in Vivo* and *in Vitro*: 4-S-Cysteaminylphenol Binds Protein Disulphide Isomerise," Melanoma Research, Vol. 1, No. 2, 1992, pp. 97-104. doi:10.1097/00008390-199106000-00004

[58] V. Hariharan, J. Klarquist, M.J. Reust, A. Koshoffer, M.D. McKee, R.E. Boissy and I.C. Le Poole, "Monobenzyl Ether of Hydroquinone and 4-Tertiary Butyl Phenol Activate Markedly Different Physiological Responses in Melanocytes: Relevance to Skin Depigmentation," *Journal of Investigative Dermatology*, Vol. 130, 2010, pp. 211-220. doi:10.1038/jid.2009.214

[59] J. G. van den Boorn, D. I. Picavet, P. F. van Swieten, H. A. van Veen, D. Konijnenberg, P. A. van Veelen, T. van Capel, E. C. Jong, E. A. Reits, J. W. Drijfhout, J. D. Bos, C. J. Melief and R. M. Luiten, "Skin-Depigmenting Agent Monobenzone Induces Potent T-Cell Autoimmunity toward Pigmented Cells by Tyrosinase Haptenation and Melanosome Autophagy," *Journal of Investigative Dermatology*, Vol. 131, No. 6, 2011, pp. 1240-1251.

[60] O. J. Finn, "Molecular Origins of Cancer," *New England Journal of Medicine*, Vol. 358, 2008, pp. 2704-2711.

[61] F. S. Hodi, F. Stephen, S. J. O'Day, D. F. McDermott, R. W. Weber, J. A. Sosman, J. B. Haanen, R. Gonzalez, C. Robert, D. Schadendorf, J. C. Hassel, W. Akerley, A. J. M. van den Eertwegh, J. Lutzky, P. Lorigan, J. M. Vaubel, G. P. Linette, D. Hogg, C. H. Ottensmeier, C. Lebbé, C. Peschel, I. Quirt, J. I. Clark, J. D. Wolchok, J. S. Weber, J. Tian, M. J. Yellin, G. M. Nichol, A. Hoos and W. J. Urba, "Improved Survival with Ipilimumab in Patients with Metastatic Melanoma," *New England Journal of Medicine*, Vol. 363, No. 8, 2010, pp. 711-723. doi:10.1056/NEJMoa1003466

[62] D. J. Schwartzentruber, D. H. Lawson, J. M. Richards, R. M. Conry, D. M. Miller, J. Treisman, F. Gailani, L. Riley, K. Conlon, B. Pockaj, K. L. Kendra, R. L. White, R. Gonzalez, T. M. Kuzel, B. Curti, P. D. Leming, E. D. Whitman, J. Balkissoon, D. S. Reintgen, H. Kaufman, F. M. Marincola, M. J. Merino, S. A. Rosenberg, P. Choyke, D. Vena and P. Hwu, "gp100 Peptide Vaccine and Interleukin-2 in Patients with Advanced Melanoma," *New England Journal of Medicine*, Vol. 364, No. 22, 2011, pp. 2119-2127. doi:10.1056/NEJMoa1012863

[63] C. Robert, L. Thomas, I. Bondarenko, S. O'Day, J. W. C. Garbe, C. Lebbe, J. F. Baurain, A. Testori, J. J. Grob, N. Davidson, J. Richards, M. Maio, A. Hauschild, W. H. Miller Jr., P. Gascon, M. Lotem, K. Harmankaya, R. Ibrahim, S. Francis, T. Chen, R. Humphrey, A. Hoos and J. D. Wolchok, "Ipilimumab Plus Dacarbazine for Previously Untreated Metastatic Melanoma," *New England Journal of Medicine*, Vol. 364, No. 26, 2011, pp. 2517-2526. doi:10.1056/NEJMoa1104621

[64] P. B. Chapman, A. Hauschild, C. Robert, J. B. Haanen, P. Ascierto, J. Larkin, R. Dummer, C. Garbe, A. Testori, M. Maio, D. Hogg, P. Lorigan, C. Lebbe, T. Jouary, D. Schadendorf, A. Ribas, S. J. O'Day, J. A. Sosman, J. M. Kirkwood, A. M. M. Eggermont, B. Dreno, K. Nolop, J. Li, B. Nelson, J. Hou, R. J. Lee, K. T. Flaherty and G. A. McArthur, "Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation," *New England Journal of Medicine*, Vol. 364, No. 26, 2011, pp. 2507-2516. doi:10.1056/NEJMoa1103782

[65] K. Mise, N. Kan, T. Okino, M. Nakanishi, K. Satoh, Y. Teramura, S. Yamasaki, K. Ohgaki and T. Tobe, "Effect of Heat Treatment on Tumor Cells and Antitumor Effector Cells," *Cancer Research*, Vol. 50, No. 19, 1990, pp. 6199-6202.

[66] A. Ito, F. Matsuoka, H. Honda and T. Kobayashi, "Heat Shock Protein 70 Gene Therapy Combined with Hyperthermia Using Magnetic Nanoparticles," *Cancer Gene Therapy*, Vol. 10, No. 12, 2003, pp. 918-925. doi:10.1038/sj.cgt.7700648

[67] M. Yanase, M. Shinkai, H. Honda, T. Wakabayashi, J. Yoshida and T. Kobayashi, "Intracellular Hyperthermia for Cancer Using Magnetite Cationic Liposomes: An *in Vivo* Study," *Cancer Science*, Vol. 89, No. 4, 1998, pp. 463-469. doi:10.1111/j.1349-7006.1998.tb00586.x

Abbreviations

AMF = alternating magnetic field
BSO = buthionine sulfoximine
CML = cationic magneto-liposome
CTI therapy = chemo-thermo-immunotherapy
DC = dendritic cell
DDS = drug delivery system
HSP/Hsp = heat shock protein
mAb = monoclonal antibody
MC1R = melanocortin 1 receptor
MITF = microphthalmia transcription factor
ML =non-cationic magneto-liposome
MSH = melanocyte stimulating hormone
NAcCAP = N-acetyl 4S-cysteaminylphenol
NPrCAP = N-propionyl 4S cysteaminylphenol
NPrCAP/M = N-propionyl 4S-cysteaminylphenol/ magnetite nanoparticle
NPrCAP/PEG/M = N-propionyl 4S-cysteaminylphenol/ polyethylene glycol/ magnetite nanoparticle
OVA = ovalbumin
PEG = polyethylene glycol
TIL = tumor infiltrating lymphocytes

メラノーマ形質を分子標的とした
ナノメデシン化学・温熱・免疫療法の基礎と臨床

皮膚病総合医学研究所　神保　孝一

転移性メラノーマに対し、メラニン形成を分子標的とした新規温熱療法である「化学・温熱・免疫療法；chemo-thermo-immunotherapoy (CTI療法)」確立の基礎と臨床を紹介する。

メラニン形成酵素、チロシナーゼの特異的基質であるチロシン（のアミン誘導体（NPrCAP, NAcCAP）を合成した。NPrCAP, NAcCAPはメラノーマ細胞に選択的に取り込まれ、チロシナーゼと反応し細胞障害性ラジカル（酸化ストレス）を産生し、メラノーマの増殖抑制を示すが、この選択的化学療法効果を増加させるために微細鉄粒子表面にNPrCAPを重合させた薬剤（NPrCAP/M）を合成し、その後交換磁場照射により温熱を発生させ選択的温熱細胞殺効果を起こさせ、結果として生じる熱ショック蛋白（HSP）とメラノーマペプチド結合体を介した温熱免疫療法により、遠隔転移メラノーマの消滅を図った。

ＣＴＩ療法の基礎的動物実験結果と製剤の合成及び実際の臨床試験の治療効果につき紹介する。

共同研究者：
高田　知明[1)]、佐藤　牧人[1)]、佐藤亜紀子[1)]、神谷　崇文[1)]、小野　一郎[1)]、山下　利春[1)]、田村　保明[2)]、佐藤　昇志[2)]、宮本　篤[2)]、若松　一雅[4)]、伊藤　祥輔[4)]、井藤　彰[5)]、本多　裕之[6)]、村瀬　勝俊[7)]
1）札幌医科大学医学部皮膚科学講座、2）札幌医科大学医学部病理学第一講座、3）札幌医科大学医学部医療薬学、4）藤田保健衛生大学衛生学部　微量成分分析学教室、5）九州大学大学院工学研究院　化学工学部門分子・生物システム工学講座、6）名古屋大学　大学院工学研究科　化学・生物工学専攻　生物機能工学分野、7）名糖産業株式会社　名古屋研究所

N-Propionyl-Cysteaminylphenol Suppresses Re-Challenge of Mouse B16F1 Tumor by Inducing Tumor-Specific Immune Response

Yasue OSAI[1], Yasuaki TAMURA[2], Noriyuki SATO[2], Kazumasa WAKAMATSU[3], Shosuke ITO[3], Akira ITO[4], Hiroyuki HONDA[5], Masae OHKURA[1], Toshiharu YAMASHITA[1], Kowichi JIMBOW[1]

Departments of [1]Dermatology and [2]1st Pathology, Sapporo Medical University, Sapporo; [3]Department of Chemistry, Fujita Health University Toyoake; [4]Department of Chemical Engineering, Kyushu University, Fukuoka; 5Department of Biotechnology, Nagoya University, Nagoya, Japan

We have previously shown that N-propionyl-cysteaminylphenol (NPrCAP) is a good substrate for tyrosinase, selectively incorporated into melanoma tissues, and inhibits the growth of melanoma cells. In the present study, we examine whether NPrCAP can suppress transplanted re-challenged secondary mouse B16F1 tumors by inducing melanoma-specific host immune responses. From the 8th day after first, primary transplantation , mice bearing B16F1 melanoma received three or five administrations of 24.4 mM NPrCAP into their melanoma tissues every other day. On the 14th day after NPrCAP administrations commenced, residual tumors were removed and B16F1 and RMA T-cell lymphoma cells were re-transplanted on the opposite sides of the flanks. Results indicated that the growth of primary as well as secondary B16F1 tumors was significantly suppressed in mice treated with NPrCAP. Growth of RMA T-cell lymphoma transplanted after the excision of the B16F1 tumor was not affected by the NPrCAP administrations. Anti-CD8 but not anti-CD4 antibody , given before and after the secondary transplantation of B16F1 cells, did not suppress the growth of these cells. Furthermore, B16F1 cells, when cultured in an NPrCAP-containing medium, showed evident sub-G1 fraction with an activation of caspase 3. These results suggest that NPrCAP has an anti-melanoma growth effect by causing apoptotic cell death that results in induction of tumor-specific host immunity consisting of CD8+ T cells. Thus it is proposed that NPrCAP may be applicable to the development of a novel melanoma-targeted therapy.

Melanogenesis substrate, N-propionyl cysteaminyl-phenol is selectively incorporated into melanoma cells and inhibits the growth of re-challenged secondary transplantation

Jimbow, K[1,3]. Thomas, PD[2]. Osai, Y[3]. Takada, T[3]. Sato, M[3]. Sato, A[3]. Tamura, T[4].

1)Inst. of Dermatology & Cutaneous Sciences, Japan
2)Div. of Dermatology & Cutaneous Sciences, University of Alberta, Canada
3)Dept. of Dermatology and 4)Pathology, Sapporo Medical University, School of Medicine, Japan

Management of metastatic melanoma is a difficult challenge for both basic scientists and clinicians. Currently available therapeutic approaches including chemotherapy, radiotherapy and immunotherapy are of limited value. Exploitation of biologic property unique to melanoma may, however, still be a challenge for developing a novel approach in solving this difficult problem. Based upon the thermo-immunotherapeutic effect of magnetite by exposing to alternating magnetic field (AMF), we recently introduced a concept for developing chemo-thermo-immunotherapy (CTI therapy) for metastatic melanoma (Jimbow et al, Pigment Cell & Melanoma Res, 21: 243, 2008). In this approach a melanogenesis substrate, N-propionyl cysteaminylphenol (NPrCAP) was conjugated with magnetite nanoparticles and exposed to AMF. This study investigated to what extent and how NPrCAP plays a novel biological role in CTI therapy. Specifically we were interested in identifying the mechanism for the selective uptake and immunotherapeutic effect of NPrCAP. The in vitro study using competitive inhibition of DNA synthesis by incorporation inhibitors showed that NPrCAP takes a selective uptake by melanoma cells through a process common to NAcCAP, DTT, cystamine and mercaptoethanol. The in vivo study using re-challenged B16 F1 and F10 melanoma after NPrCAP treatment showed that NPrCAP alone can suppress the transplanted secondary tumor through melanoma-specific host immune response.

Melanogenesis cascade for developing novel selective drug delivery and chemo-thermo-immunotherapeutic strategies in melanoma; specificity and biological effect

Jimbow K[1),3)], Thomas PD[2)], Osai Y[3)], Takada T[3)], Sato M[3)], Sato A[3)], Tamura Y[4)], Kamiya T[3)], Ono I[3)], Yamashita T[3)], Ito A[5)], Honda H[6)], Wakamatsu K[7)], Ito S[7)]

1) Institute of Dermatology & Cutaneous Sciences, Japan

2) Division of Dermatology & Cutaneous Sciences, University of Alberta, Canada

3) Department of Dermatology and 4)Pathology, Sapporo Medical University, School of Medicine, Japan

5) Department of Chemical Engineering, Faculty of Engineering, Kyushu University

6) Bioprocess Engineering Laboratory, Department of Biotechnology School of Engineering, Nagoya University

7) Department of Chemistry, Fujita Health University School of Health Sciences

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. N-propionyl 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. Based upon these unique biological properties, we now provide evidence that the melanogenesis cascade can be exploited for developing a novel chemo-thermo-immunologic approach (CTI/Therapy) for melanoma by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/M). Here in this study we investigated to what extent and how NPrCAP plays a novel biological role in CTI therapy. Specifically we were interested in identifying the mechanism for the selective uptake and immunotherapeutic effect of NPrCAP. The in vitro study using competitive inhibition of DNA synthesis by incorporation inhibitors showed that NPrCAP takes a selective uptake by melanoma cells through a process common to NAcCAP, DTT, cystamine and mercaptoethanol. The in vivo study using re-challenged B16 F1 and F10 melanoma after NPrCAP treatment showed that NPrCAP alone can suppress the transplanted secondary tumor through melanoma-specific host immune response.

N-propionyl-4-S-cysteaminylphenol induces apoptosis of mouse B16F1 melanoma cells and suppression of transplanted B16F1 tumors

Y. Osai, Y. Tamura, N. Sato, K. Wakamatsu, S. Ito, A. Ito,
H. Honda, M. Okura, T. Yamashita, K. Jimbow

Departments of Dermatology and 1st Pathology, Sapporo Medical University, Sapporo; Department of Chemistry, Fujita Health University Toyoake; Department of Chemical Engineering, Kyushu University, Fukuoka; Department of Biotechnology, Nagoya University, Japan

Melanogenesis is a differentiation phenotype specific for melanocytes and most melanoma cells, and produces reactive oxygen species that cause the deterioration of melanoma cells. We studied a mechanism of death of melanoma cells induced by N-propionyl-4-S-cysteaminylphenol (NPrCAP), and examined whether NPrCAP can induce the suppression of primary and re-challenged mouse B16F1 tumors. When mouse B16F1 cells were cultured in the NPrCAP-containing medium, evident sub-G1 fraction was observed and the cell extract contained activated caspase 3. When mice bearing B16F1 melanoma received intra-tumoral administrations of NPrCAP, a decrease in tumor sizes was observed. After primary tumor was removed on the 14th day, B16F1 cells were re-transplanted. Growth of the secondary tumors was significantly suppressed. Tumors on mice that received anti-CD8 mAb grew similarly as those in the non-treated mice. These results suggest that (1) NPrCAP has cytotoxicity causing apoptotic cell death, and (2) it induces tumor-specific host immunity consisting of CD8+ T cells in the model animal. Thus, NPrCAP is applicable to the novel treatment by both the induction of apoptosis and CD8(+)-mediated cell immunity in human melanoma.

Utilization of melanogenesis substrate, NPrCAP for exploiting melanoma-targeting drug and its conjugation with magnetite nanoparticles for developing melanoma chemo-thermo-immunotherapy.

Kowichi Jimbow[1,6], Tomoaki Takada[1], Makito Sato[1], Akiko Sato[1], Yasue Osai[1], Takafumi Kamiya[1], Ichiro Ono[1], Toshiharu Yamashita[1], Yasuaki Tamura[2], Akira Ito[3], Hiroyuki Honda[4], Kazumasa Wakamatsu[5] and Shosuke Ito[5]

Departments of [1]Dermatology and [2]Pathology 1, Sapporo Medical University School of Medicine
[3]Department of Chemical Engineering, Faculty of Engineering, Kyushu University; [4]Department of Biotechnology, School of Engineering, Nagoya University
[5]Department of Chemistry, Fujita Health University School of Health Sciences
[6]Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences

E-mail address: jimbow@sapmed.ac.jp
Affiliation: Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences
Mailing Address: Sapporo Medical Care Centre 4th Floor, Chuoku, Odori, West 17, 11-23, Sapporo, Japan 060-0042
TEL: 81－11－887－8266 / FAX: 81－11－618－1213

Exploitation of a specific biological property is one of the best approaches for developing novel cancer targeted drugs. Melanogenesis substrate, N-propionyl cysteaminylphenol (NPrCAP) may provide a novel drug delivery system because of its selective incorporation into melanoma cells as well as act as a melanoma targeted drug because of its production of highly reactive free radicals (melanoma targeted chemotherapy). Utilization of magnetite nanoparticles can also be a good platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to an alternating magnetic field (AMF). This study shows the feasibility of this approach in experimental study using in vivo and in vitro B16 melanoma cells and preliminary clinical study with a limited number of advanced melanoma patients. The therapeutic protocol against the primary transplanted tumor with or without AMF once a day every other day for a total of three treatments not only inhibited the growth of primary transplant, but also prevented the growth of the secondary, re-challenge transplant and increased life span of the host mice. The heat-generated therapeutic effect was more significant at a temperature of 43℃ than either 41℃ or 46℃. HSP70 production at the site of primary transplant and CD8+T cell infiltration at the site of the re-challenge melanoma transplant were seen. Four patients entered in the preliminary clinical trial by following the basic outline of this animal protocol and two of them showed PR and CR.

Conjugation of NPrCAP, melanogenesis substrate and magnetite nanoparticles with alternating magnetic field can provide a novel melanoma chemo-thermo-immunotherapy; feasibility, specificity and preliminary clinical efficacy

Kowichi JIMBOW[1,6], Yasue Osai[1], Akiko Sato[1], Tomoaki Takada[1], Makito Sato[1], Takafumi Kamiya[1], Ichiro Ono[1], Toshiharu Yamashita[1], Yasuaki Tamura[2], Akira Ito[3], Hiroyuki Honda[4], Kazumasa Wakamatsu[5], Shosuke Ito[5] , Eiichi Nakayama

Departments of [1]Dermatology and [2]Pathology 1, Sapporo Medical University School of Medicine

[3]Department of Chemical Engineering, Faculty of Engineering, Kyushu University; [4]Department of Biotechnology, School of Engineering, Nagoya University

[5]Department of Chemistry, Fujita Health University School of Health Sciences

[6]Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences

E-mail address: jimbow@sapmed.ac.jp

Affiliation: Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences

Mailing Address: Sapporo Medical Care Centre 4th Floor, Chuoku, Odori, West 17, 11-23, Sapporo, Japan 060-0042

TEL: 81-11-887-8266 / FAX: 81-11-618-1213

NPrCAP (N-propionyl cysteaminylphenol) is a unique substrate for melanin biosynthesis substrate in melanoma. It is selectively incorporated into melanoma, providing a unique melanoma-targeted drug delivery system, and disintegrates melanoma cells through highly reactive free radicals produced by interaction with tyrosinase, hence being able to act as a melanoma-targeted chemotherapy agent. Utilization of magnetite nanoparticles can also be a good platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to an alternating magnetic field (AMF). This study introduces our protocol of this combined approach in conjugation of NPrCAP and magnetite nanoparticles with exposure to AMF, and reports the feasibility and specificity of the

approach in experimental study using *in vivo* and *in vitro* B16 melanoma cells. In addition a preliminary clinical study with a limited number of advanced melanoma patients is shown. The therapeutic protocol against the primary transplanted tumor with or without AMF once a day every other day for a total of three treatments not only inhibited the growth of primary transplant, but also prevented the growth of the secondary, re-challenge transplant and increased life span of the host mice. Importantly NPrCAP alone could produce growth inhibition of re-challenge melanoma transplant. HSP production at the site of primary transplant and $CD8^{+}$T cell infiltration at the site of the re-challenge melanoma transplant were seen. Four patients entered in the preliminary clinical trial by following the basic outline of this animal protocol and two of them showed PR and CR. It is concluded that the utilization of a biological property of melanoma, i.e., melanogenesis which is highly and uniquely expressed, can be employed in developing highly reactive, melanoma-targeted drug, acting as both chemotherapeutic and immunogenic drug, and that a combined therapy of NPrCAP and magnetite nanoparticles can provide a basis for establishing "chemo-thermo-immuno-therapy CTI therapy for melanoma.,

Melanogenesis substrate, N-propionyl cysteaminylphenol, alone possesses a potent chemotherapeutic and immunogenic property in conducting melanoma chemo-thermo-immunotherapy, a combined therapy of NPrCAP and magnetite nanoparticles with HSP generation by alternating magnetic field.

Kowichi JIMBOW[1,6], Yasue OSAI[1], Akiko SATO[1], Tomoaki TAKADA[1], Makito SATO[1], Takafumi Kamiya[1], Ichiro ONO[1], Toshiharu YAMASHITA[1], Yasuaki TAMURA[2], Akira ITO[3], Hiroyuki HONDA[4], Kazumasa WAKAMATSU[5], Shosuke ITO[5] , Takeshi KOBAYASHI and Eiichi NAKAYAMA

Departments of [1]Dermatology and [2]Pathology 1, Sapporo Medical University School of Medicine

[3]Department of Chemical Engineering, Faculty of Engineering, Kyushu University;

[4]Department of Biotechnology, School of Engineering, Nagoya University

[5]Department of Chemistry, Fujita Health University School of Health Sciences

[6]Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences

E-mail address: jimbow@sapmed.ac.jp

Affiliation: Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences

Mailing Address: Sapporo Medical Care Centre 4th Floor, Chuoku, Odori, West 17, 11-23, Sapporo, Japan 060-0042

TEL: 81-11-887-8266 / FAX: 81-11-618-1213

Melanogenesis substrate, N-propionyl cysteaminylphenol (NPrCAP) provides a novel drug delivery system because of its selective incorporation into melanoma cells and act as a melanoma-targeted drug because of its production of highly reactive free radicals (melanoma targeted chemotherapy). Utilization of magnetite nanoparticles can also be a good platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to an alternating magnetic field (AMF). We have evaluated the feasibility of this approach in experimental study using *in vivo* and *in vitro* B16 melanoma cells. Using this strategy called "chemo-thermo-immuno (CTI) therapy", we were able to show CR and PR results in a pilot preliminary clinical study with a limited number of patients. This study reports to what extent and at which site of CTI therapy NPrCAP alone exerts its effect. The therapeutic protocol against the primary transplanted tumor with or without AMF once a day every other day for a total of three treatments not only inhibited the growth of primary transplant, but also prevented

the growth of the secondary, re-challenge transplant and increased life span of the host mice. HSP production at the site of primary transplant and $CD8^{+}$T cell infiltration at the site of the re-challenge melanoma transplant were seen. Importantly NPrCAP alone could produce growth inhibition of re-challenge melanoma transplant. Specifically "first B16 melanoma transplanted" mice treated by *ip* administration of NPrCAP for 3-5times showed not only growth inhibition of primary transplant but also the marked and significant growth inhibition of "second re-challenge melanoma". The latter immunologic rejection was blocked by anti-CD8+ Ab, but not by CD4+ AB. The finding indicates that NPrCAP alone has both chemo-and immunotherapeutic property in CTI therapy, hence it could be a good candidate for melanoma adjuvant therapy.

Abstract

Melanoma Targeted Chemo-Thermo-Immunotherapy, A combined Therapy of NPrCAP and magnetite nanoparticles with HSP generation

Kowichi JIMBOW[1,9], Yasue OSAI[1], Akiko SATO[1], Tomoaki TAKADA[1], Makito SATO[1], Takafumi Kamiya[1], Ichiro ONO[1], Toshiharu YAMASHITA[1], Yasuaki TAMURA[2], Akira ITO[3], Hiroyuki HONDA[4], Kazumasa WAKAMATSU[5], Shosuke ITO[5] , Satoshi NOHARA[6], Takeshi KOBAYASHI[7] and Eiichi NAKAYAMA[8]

Departments of [1]Dermatology and [2]Pathology 1, Sapporo Medical University School of Medicine,

[3]Department of Chemical Engineering, Faculty of Engineering, Kyushu University,

[4]Department of Biotechnology, School of Engineering, Nagoya University,

[5]Department of Chemistry, Fujita Health University School of Health Sciences,

[6]Meitou Seika Inc,

[7]Department of Immunology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences,

[8]Department of Biological Chemistry, College of Bioscience and Biotechnology, Chubu University,

[9]Institute of Dermatology & Cutaneous Sciences,

Japan

E-mail address: jimbow@sapmed.ac.jp

Affiliation: Department of Cutaneous Sciences, Institute of Dermatology & Cutaneous Sciences

Mailing Address: Sapporo Medical Care Centre 4th Floor, Chuoku, Odori, West 17, 11-23, Sapporo, Japan 060-0042

TEL: 81-11-887-8266 / FAX: 81-11-618-1213

Introduction: N-propionyl cysteaminylphenol (NPrCAP) is a compound that is tyrosinase substrate and selectively incorporates into melanoma cells and disintegrate melanoma cells through production of highly reactive free radicals by interaction with tyrosinase. It will thus provide a novel drug delivery system and act as a melanoma targeting chemotherapeutic drug. Utilization of magnetite nanoparticles can also be a good platform to develop thermo-immunotherapy because of heat shock protein (HSP) generation upon exposure to an alternating magnetic field (AMF).

Objective and Purpose: We have evaluated the efficacy of the combination therapy of NPrCAP and magnetite nanoparticles by experimental studies using *in vivo* and *in vitro*