

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

cells (Shellman *et al.*, 2007). Intracellular hyperthermia of NPrCAP/M-treated cells resulted in a significant degradation of melanoma cells (Figure 5a). No difference was found in the cell numbers of non-melanoma cell lines between NPrCAP/M- and magnetite-treated dishes (Figure 5b). These results were comparable to those of an iron-incorporation assay of cells cultured in NPrCAP/M- and magnetite-containing media (Figure 3), suggesting that the targeting ability of NPrCAP/M to melanocytic cells determined the degree of cell degradation. In a previous study, significantly higher therapeutic effects were observed in mice treated with 4-S-CAP/MCL or hyperthermia alone (Ito *et al.*, 2007). In our animal-model experiments, growth of transplanted B16F1 tumors was suppressed more effectively than in the untreated control. Because untreated control mice died on the 19th and 21st days after inoculation, survival curves over a period of 60 days could not be compared. However, two and one mice that received NPrCAP/M- and magnetite-mediated hyperthermia survived to the 60th day after treatment, respectively, whereas mice belonging to the other groups did not. Therefore, NPrCAP/M- and magnetite-mediated hyperthermia was suggested to be more effective for tumor suppression

than treatment without heat. Interestingly, NPrCAP/M injection without heat suppressed B16 melanoma more than in the control mice (Figure 6b), suggesting that NPrCAP possessed intrinsic cytotoxicity against melanoma cells. Another experimental system that can evaluate targeting, permeating, and suppressive abilities of NPrCAP/M needs to be designed.

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

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

MATERIALS AND METHODS

Cell lines and cell culture

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

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

Establishment of tumors in mice

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

Animal treatment model

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

Iron oxide and chemicals

Magnetite nanoparticles (Fe_3O_4 ; average particle size, 10 nm) were kindly provided by Toda Kogyo (Hiroshima, Japan). 4-S-CAP was prepared as described by Padgett *et al.* *N*-succinimidyl-3-[2-pyridyl]dithio] propionate and mushroom tyrosinase (6050 U mg^{-1}) were obtained from Molecular Biosciences, Inc. (Boulder, CO) and Sigma Chemical Co. (St Louis, MO), respectively. 3-Aminopropyltriethoxysilane and *N*-[γ -maleimidobutyryloxy]sulfo succinimide ester were products of Tokyo Chemical Industry (Tokyo, Japan) and Pierce (Rockford, IL), respectively. All other chemicals were of analytical grade.

Synthesis of *N*-(1-mercaptopropionyl)-4-S-cysteamylphenol (NPrCAP-SH)

A mixture of 1.81 g of 4-S-CAP (10.7 mmol) and 4.13 g of *N*-succinimidyl-3-[2-pyridyl]dithio] propionate (13.2 mmol) in 5 ml of pyridine was stirred for 2 hours at room temperature. After evaporation, the residue was purified by silica gel column chromatography (ethyl acetate:n-hexane; 2:1 v/v as eluant) to give a disulfide (3.70 g; 94%). Then 4.29 g of dithiothreitol (27.5 mmol) was added to a stirred solution of the disulfide (3.70 g; 10.3 mmol) in

5 ml of methanol at room temperature. After 2 hours the mixture was evaporated and the oily residue was purified by silica gel column chromatography (ethyl acetate:n-hexane; 2:1 v/v) to give 2.19 g of NPrCAP-SH (80%) as a colorless crystal after recrystallization (ethyl acetate ether). The elemental analysis of NPrCAP-SH was as follows: Anal. Calcd for $\text{C}_{11}\text{H}_{15}\text{N}_1\text{O}_2\text{S}_2$: C, 51.36; H, 5.84; N, 5.45; S, 24.90; Found: C, 51.41; H, 5.78; N, 5.50; S, 24.83. The resulting material was subjected to liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis using an electrospray ionization/ion trap mass spectrometer (LCQ Deca XP, Thermolectron, Tokyo, Japan). The analysis was carried out directly by MS/MS at a positive charge; $[\text{M} + \text{H}]^+$: m/z 258, 164, 153, 132, 125. ^1H NMR was measured at 400 MHz (CD_3COCD_3): 2.45 p.p.m. (2H, t, $J=6.8$ Hz), 2.71 p.p.m. (2H, m), 2.88 p.p.m. (2H, t, $J=6.3$ Hz), 3.32 p.p.m. (2H, m), 6.68 p.p.m. (2H, d, $J=8.0$ Hz), 7.31 p.p.m. (2H, d, $J=8.0$ Hz).

Conjugation of NPrCAP-SH with magnetite

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

Analysis of NPrCAP incorporated with magnetite nanoparticles

The degree of incorporation of NPrCAP-SH with magnetite was determined by hydrolysis with 6 M HCl followed by HPLC analysis of the resultant 4-S-CAP. Briefly, the amount of 4-S-CAP produced by the hydrolysis of NPrCAP/M with 6 M HCl at 110°C for 1.5 hours was measured by HPLC using a Jasco PU-980 Intelligent liquid chromatogram with a Jasco 851-AS Intelligent autosampler (JASCO, Tokyo, Japan), a Jasco 875-UV/VIS detector, and Shiseido C18 reverse-phase column (Capcell Pak C18, $4.6 \times 250 \text{ mm}$; $5.0 \mu\text{m}$ particle size). The UV detector was set at 250 nm. The mobile phase used was methanol: H_2O :1.0 M HClO_4 , 10:90:1.5 by volume. The analyses were performed at 50°C at a flow rate of $0.7 \text{ ml minute}^{-1}$. The concentration of iron, which formed a red complex with thiocyanate, was quantitated by absorbance at 480 nm (Owen and Sykes, 1984).

Tyrosinase oxidation of NPrCAP/M

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

HClO₄. The concentrations of the substrate remaining as 4-S-CAP and 5-S-cysteaminy-3-S'-cysteinylcatechol produced were measured by hydrolysis with 6 M HCl followed by HPLC analysis (see above section, Analysis of NPrCAP incorporated with magnetic particles). The retention times of 4-S-CAP and 5-S-cysteaminy-3-S'-cysteinylcatechol were 14.4 and 7.9 minutes, respectively.

Measurement of iron in the NPrCAP/M-exposed cells

Subconfluent growing melanoma and non-melanoma cells ($8 \times 10^4 \text{ cm}^{-2}$) in a 25 cm² flask were refed with the medium containing 5.94 mg of NPrCAP/M or magnetite ($84 \mu\text{g ml}^{-1}$). To discriminate between incorporation of NPrCAP/M by direct attachment to cells and that by diffusion from the medium, culture flasks were fixed on a slanted disc (60°) and rotated slowly for 30 minutes. After the cells were washed with PBS twice and collected, they were dissolved completely in 200 μl of concentrated HCl and incubated at 43°C for 30 minutes. Then, 10 μl of H₂O₂ and 4 ml of 1% potassium thiocyanate were added in sequence to the cell solution. The iron concentration of magnetite nanoparticles was measured using the potassium thiocyanate method as described above.

Cytotoxicity of NPrCAP/M in combination with AMF irradiation

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

Recombinant adenoviruses

Propagation, plaque formation, and inoculation of recombinant adenoviruses were described elsewhere (Yamano *et al.*, 1999). Ad-LacZ is a replication-deficient recombinant adenovirus carrying β -galactosidase. Ad-p63' is a recombinant adenovirus expressing modified p63, which was provided by T Tokino (manuscript in preparation and personal communication). The construction of the original adenovirus Ad-p63 containing the human p63 gene was described previously (Sasaki *et al.*, 2001, 2003). Cells were infected with 20 plaque-forming units of a recombinant adenovirus, incubated for 60 minutes at 37°C, and cultured in fresh DMEM with 5% FBS for 48 hours before flow cytometric analysis or caspase assay.

Flow cytometric analysis

After B16F1 and SK-mel-23 cells were cultured in the NPrCAP/M-containing medium (4.6 mg ml^{-1}) for 30 minutes, they were subjected to AMF irradiation at 43°C for 30 minutes, reseeded in the medium, and cultured for 24 hours. Then adherent and floating cells were collected together and washed in ice-cold PBS. The cells were dehydrated in 75% cold ethanol and stored on ice for 2 hours. Then

they were rehydrated in cold PBS and incubated in the presence of RNaseA ($50 \mu\text{g ml}^{-1}$) (Sigma Aldrich Japan, Tokyo, Japan) at 37°C for 30 minutes. After incubation, the cells were rinsed twice in ice-cold PBS and suspended in 2.0 ml of PBS containing $50 \mu\text{g ml}^{-1}$ propidium iodide (Sigma Aldrich Japan) at 4°C for 2 hours. Cell debris and fixation artifacts were gated out, and sub-G1, G1, S, and G2/M populations were quantified with a FACScan Cell Sorter (Nippon Becton Dickinson, Tokyo, Japan) using the CELL QUEST program.

Caspase enzyme assay

After 5×10^6 B16F1 and SK-mel-23 cells were collected in 15 ml tubes, 5.94 mg of NPrCAP/M was separately added to the cell pellets, which were then incubated for 20 minutes at 37°C in a CO₂ incubator. Cells were harvested by centrifugation at 400 g for 10 minutes and irradiated by the AMF as described above. Cells were then collected and seeded in 10 cm dishes and cultured for a further 24 hours. Both the floating and the adherent cells were collected, washed with PBS, and processed for caspase assay. Activities of caspases 3, 8, and 9 were measured using a colorimetric protease kit according to the manufacturer's protocol (MBL, Nagoya, Japan).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

- Alena F, Iwashina T, Gili A, Jimbow K (1994) Selective *in vivo* accumulation of *N*-acetyl-4-S-cysteaminyphenol in B16F10 murine melanoma and enhancement of its *in vitro* and *in vivo* antimelanoma effect by combination of buthionine sulfoximine. *Cancer Res* 54:2661-6
- Balch CM, Buzaid AC, Soong S-J, Atkins MB, Cascinelli N, Coit DG *et al.* (2001) Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* 19:3635-48
- Brostrom CO, Brostrom MA (1998) Regulation of translational initiation during cellular responses to stress. *Prog Nucleic Acid Res Mol Biol* 58:79-125
- Han Q-S, Yumita N, Nishigaki R (2007) Involvement of caspases 3, 8, and 9 signaling pathways in hyperthermia induced apoptosis in HL-60 cells. *Thermal Med* 23:31-40
- Ito A, Fujioka M, Yoshida T, Wakamatsu K, Ito S, Yamashita T *et al.* (2007) 4-S-cysteaminyphenol-loaded magnetite cationic liposomes for combination therapy of hyperthermia with chemotherapy against malignant melanoma. *Cancer Sci* 98:424-30
- Ito A, Tanaka K, Kondo K, Shinkai M, Honda H, Matsumoto K *et al.* (2003) Tumor regression by combined immunotherapy and hyperthermia using magnetic nanoparticles in an experimental subcutaneous murine melanoma. *Cancer Sci* 94:308-13
- Jimbow K, Iwashina T, Alena F, Yamada K, Pankovich J, Umemura T (1993) Exploitation of pigment biosynthesis pathway as a selective chemotherapeutic approach for malignant melanoma. *J Invest Dermatol* 100:S231-8

- Jimbow K, Miura S, Ito Y, Kasuga T, Ito S (1984) Utilization of melanin precursors for experimental chemotherapy of malignant melanoma. *Jpn J Cancer Chemother* 11:2125-32
- Kawai N, Ito A, Nakahara Y, Futakuchi M, Shirai T, Honda H *et al.* (2005) Anticancer effect of hyperthermia on prostate cancer mediated by magnetite cationic liposomes and immune-response induction in transplanted syngeneic rats. *Prostate* 64:373-81
- Leary SP, Liu CY, Apuzzo ML (2006) Toward the emergence of nanoneurosurgery: part II-nanomedicine: diagnostics and imaging at nanoscale level. *Neurosurgery* 58:805-23
- Luderer AA, Borrelli NF, Panzario JN, Mansfield GR, Hess DM, Brown JL *et al.* (1983) Glass-ceramic-mediated, magnetic-field-induced localized hyperthermia: response of a murine mammary carcinoma. *Radiat Res* 94:190-8
- Minamimura T, Sato H, Kasaoka S, Saito T, Ishikawa S, Takemori S *et al.* (2000) Tumor regression by inductive hyperthermia combined with hepatic embolization using dextran magnetite-incorporated microspheres in rats. *Int J Oncol* 16:1153-8
- Minamitsuji Y, Toyofuku K, Sugiyama S, Yamada K, Jimbow K (1999) Sulfur containing tyrosine analogs can cause selective melanocytotoxicity involving tyrosinase-mediated apoptosis. *J Invest Dermatol Symp Proc* 4:130-6
- Miura T, Jimbow K, Ito S (1990) The *in vivo* antimelanoma effect of 4-S-cysteaminylphenol and its N-acetyl derivative. *Int J Cancer* 46:931-4
- Owen CS, Sykes NL (1984) Magnetic labeling and cell sorting. *J Immunol Methods* 73:41-8
- Padgett SR, Herman HH, Han JH, Pollock SH, May SW (1984) Anti-hypertensive activities of phenyl aminoethyl sulfides, a class of synthetic substrates for dopamine beta-hydroxylase. *J Med Chem* 27:1354-7
- Sasaki Y, Mita H, Toyota M, Ishida S, Morimoto I, Yamashita T *et al.* (2003) Identification of the interleukin 4 receptor alpha gene as a direct target for p73. *Cancer Res* 23:8145-52
- Sasaki Y, Morimoto I, Ishida S, Yamashita T, Imai K, Tokino T (2001) Adenovirus-mediated transfer of the p53 family genes, p73 and p51/p63 induces cell cycle arrest and apoptosis in colorectal cancer cell lines: potential application to gene therapy of colorectal cancer. *Gene Ther* 8:1401-8
- Shellman YG, Howe WR, Miller LA, Goldstein NB, Pacheco TR, Mahajan RL *et al.* (2008) Hyperthermia induces endoplasmic reticulum-mediated apoptosis in melanoma and non-melanoma skin cancer cells. *J Invest Dermatol* 128:949-56
- Shinkai M, Yanase M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T (1996) Intracellular hyperthermia for cancer using magnetite cationic liposomes: *in vitro* study. *Jpn J Cancer Res* 87:1179-83
- Tandon M, Thomas PD, Shokravi M, Singh S, Samra S, Chang D *et al.* (1998) Synthesis and antitumour effect of the melanogenesis-based antimelanoma agent, N-propionyl-4-S-cysteaminylphenol. *Biochem Pharmacol* 55:2023-9
- Thomas PD, Kishi H, Cao H, Ota M, Yamashita T, Singh S *et al.* (1999) Selective incorporation and specific cytotoxic effect as the cellular basis for the antimelanoma action of sulphur containing tyrosine analogs. *J Invest Dermatol* 113:928-34
- Van Vlerken LE, Amiji MM (2006) Multi-functional polymeric nanoparticles for tumour-targeted drug delivery. *Expert Opin Drug Deliv* 3:205-16
- Yamano S, Tokino T, Yasuda M, Kaneuchi M, Takahashi M, Niitsu Y *et al.* (1999) Induction of transformation and p53-dependent apoptosis by adenovirus type 5 E4orf6/7 cDNA. *J Virol* 73:10095-103
- Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T (1997) Intracellular hyperthermia for cancer using magnetite cationic liposomes: *ex vivo* study. *Jpn J Cancer Res* 88:630-2
- Yanase M, Shinkai M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T (1998) Intracellular hyperthermia for cancer using magnetite cationic liposomes: an *in vivo* study. *Jpn J Cancer Res* 89:463-9
- Yoo J, Lee YJ (2007) Effect of hyperthermia on TRAIL-induced apoptotic death in human colon cancer cells: development of a novel strategy for regional therapy. *J Cell Biochem* 101:619-30

Epidermal melanin unit and aging of skin ; biological and molecular significance of pheomelanin in constitutive photo-aging process

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Abstract

This report describes some aspects of current progress in biological and molecular involvement of melanin pigmentation in aging process of the skin. Two types of melanin pigments are present in the skin, eumelanin and pheomelanin. The importance of re-visiting the concept of epidermal melanin unit (EMU) was introduced for better understanding the role of melanin pigmentation in aging process of the skin. We then discussed what regulates in 1) biological and molecular process of melanin pigmentation in constitutive, intrinsic aging and 2) pheomelanin biosynthesis and its involvement in facultative, extrinsic aging. The former topic was further extended into a) melanogenesis and oxidative stress in EMU after exposure to UVR, b) tyrosinase and LAMP (lysosome-associated membrane protein) gene families and oxidative stress, c) function and mutation of LAMP and TYRP (tyrosinase-related protein). The latter was extended into a) high content of pheomelanin in melanoma and its precursors, b) UV photoproducts of melanin pigmentation and cytotoxicity and c) UV-photoproducts of pheomelanin in aging process. It was indicated that, while the major role of eumelanin is to provide protection against UV-induced DNA damage by absorbing and scattering UVR, pheomelanin and its precursors are photo-chemically unstable in the presence of UVR and that their oxidation products produce short-lived singlet oxygen and its conversion to hydroxyl radicals, thus affecting significantly in constitutive aging process of the skin.

Keywords : Melanin pigmentation, aging process of skin, Eumelanin, Pheomelanin, Epidermal melanin unit, Facultative pigmentation, Constitutive pigmentation.

Introduction ; Epidermal melanin unit (EMU) and melanin pigmentation in facultative and constitutive aging processes of skin

The major determinant of the skin pigmentation in humans is the epidermal melanin unit (EMU). The EMU is composed of the orderly interaction of a melanocyte and an associated pool of keratinocytes with four major biological and biochemical processes, i.e., the synthesis and melanization of melanosomes within the melanocyte, their transfer from the tip of melanocytic dendrites to surrounding keratinocytes and their degradation within keratinocytes and exfoliation from them. If there is any alteration of

these processes, hypo- or hyperpigmentation occurs and results in various skin pigmentation ranging from white, light brown, brown to black skin. If there is, however, any dropping off of melanosomes into the dermis or any growth of dermal melanocytes, slate blue or blue pigmentation occurs²⁵.

There will be the increase of skin pigmentation with natural aging of the skin and this will be typically seen in darkening of the skin in aged Orientals such as in Japanese. The increased melanin pigmentation after exposure to UVR may damage the skin and enhance the acquired skin aging as there will be increased production of free radicals during melanin formation process after exposure to UVR. The major goal of skin protection from aging process of the skin in terms of melanin pigmentation would be to repair facultative, acquired skin pigmentation, i.e., "tanning", and to reach constitutive pigmentation, i.e., the inborn skin pigmentation. If we introduce the concept of the EMU in identifying

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the methods to improve the darkening of the skin with aging, target molecules responsible for this goal may be divided into three biological and biochemical steps of pigmentation in the EMU, i.e., step 1: exfoliation of melanin/melanosome from horny layer, step 2: transfer of melanin and melanosomes from the melanocyte and their degradation within keratinocytes and finally step 3: their biosynthesis within the melanocyte (Table I).

UV irradiation, in particular UVB, is directly absorbed by cellular DNA, leading to the formation of DNA photoproducts, mainly thymidine dimers and pyrimidine (6-4) pyrimidone. Importantly the action spectrum for tanning is virtually the same as that for the formation of thymine dimers,^{14),42)} and UV induced melanogenesis can be augmented in melanocytes by treatment with T4 endonuclease V, an enzyme that acts to enhance the repair of UV-induced DNA damage^{13),41)}. Furthermore, the tumor suppressor protein p53, when activated, up-regulates the level of tyrosinase mRNA and protein, enhancing melanogenesis³³⁾. Cui et al¹⁰⁾ found a p53 consensus sequence in the proopiomelanocortin (POMC) gene promoter and established a continuous signaling pathway from UV-induced DNA injury to the final tanning response. It was shown that, following UVR in mice, p53 activation stimulates transcription from the POMC promoter in keratinocytes, thereby increasing the release of POMC-derived α MSH. Keratinocyte-derived α MSH then stimulates the melanocortin-1 receptor (MC 1 R) on melanocytes, resulting in increased production of black melanin. In contrast, p53 knockout control mice did not show POMC upregulation, increased α MSH, and subsequent

UV-induced tanning⁴¹⁾.

Mammalian melanin has been divided into two major groups, i.e., eumelanin and pheomelanin²⁹⁾. Eumelanin, brown or black, is intractably insoluble and nitrogenous. It is derived from conversion of tyrosine to form dopa and dopaquinone in the presence of tyrosinase. It is basically composed of indole-quinone units which form eumelanin by either auto-oxidation or enzymic action of tyrosinase-related proteins (TYRPs). In contrast pheomelanin is formed by the nucleophilic addition of cysteine to dopaquinone, resulting in the formation of cysteinyl-dopas, which are then auto-oxidized to form benzothiazine units and subsequently pheomelanin. Thus synthesis of both eumelanin and pheomelanin starts from tyrosine oxidation catalyzed by tyrosinase. The resulting dopaquinone can be a precursor for either eumelanin synthesis promoted by TYRP 1 and TYRP 2 (dopachrome tautomerase; DCT), or pheomelanin in the presence of high cysteine concentrations and low tyrosinase²⁵⁾. Eumelanin is the major component of the EMU, and only a small amount of pheomelanin is present in normal skin. Since both eumelanosomes and pheomelanosomes can be synthesized by a single melanocyte at the same time, it is expected that both eumelanin and pheomelanin are present in normal skin (Fig. 1 and 2)²³⁾.

The mechanism of melanin synthesis and its regulation has been extensively studied by genetic studies of coat color in mice (Fig. 3 and 4)⁶⁾. Two major loci are central to pigment-type switching (eumelanin vs pheomelanin). One is the agouti locus encoding agouti signal protein (ASIP) and other is the melanocortin-1 receptor (Mc 1 r) locus³⁾ MC 1 R (Mc 1 r) is a cell-

Table I : Molecular targets for skin pigmentation and anti-aging

Step 1 : Melanin/ melanosome exfoliation
Approach 1 : Remove horny layer cells
Approach 2 : Increase epidermal turnover
Approach 3 : Increase the contents of water/ sebum
Step 2 : Melanin/ melanosome transfer and degradation
Approach 1 : Melanosome degradation in keratinocytes
Approach 2 : Melanosome transfer in melanocytes
Step 3 : Melanin/ melanosome biosynthesis
Approach 1 : Transcription factors and MSH
Approach 2 : Tyrosinase glycosylation and molecular chaperon
Approach 3 : Vesicular transport of tyrosinase and melanization

surface G-protein-coupled receptor for which the best-known agonist is the soluble peptide α MSH, cleaved from the precursor POMC in the pituitary and skin¹²). Binding of α MSH to MC 1 R is known to activate adenylate cyclase and cAMP synthesis, promoting eumelanin synthesis through both post-translational¹⁵) and transcriptional pathways via microphthalmia-associated

transcription factor (MITF)^{7,35}). MITF is a master regulator for eumelanogenesis, melanocyte differentiation, proliferation and survival. It promotes transcription of melanocyte-specific gene products including melanosomal enzymes, tyrosinase, TYRP 1 and DCT and the matrix protein, i.e., SIVL/PMEL³⁵). ASIP competitively antagonizes α MSH at the MC 1 R and inhibits the

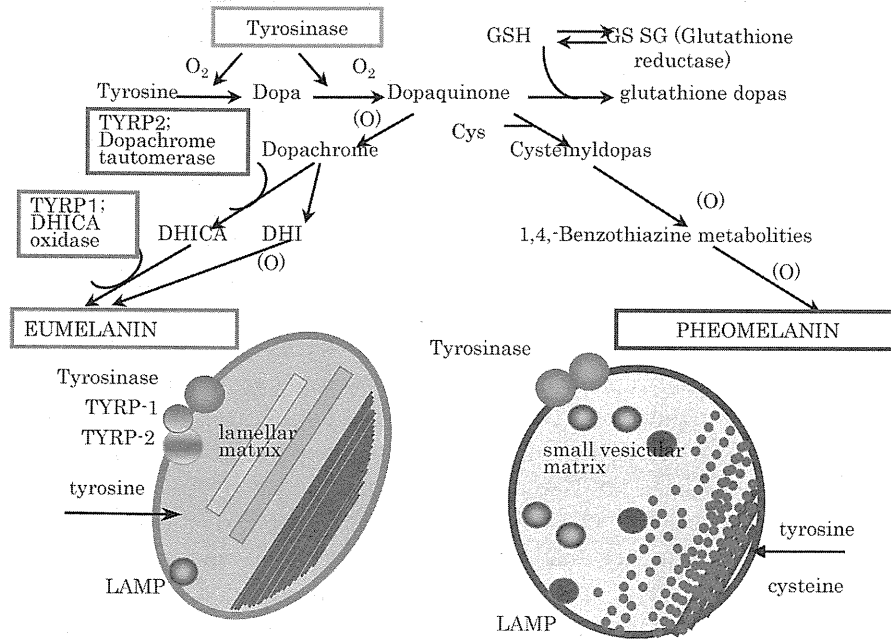


Figure 1 : Chemical and biochemical pathway for synthesis of eumelanin and pheomelanin

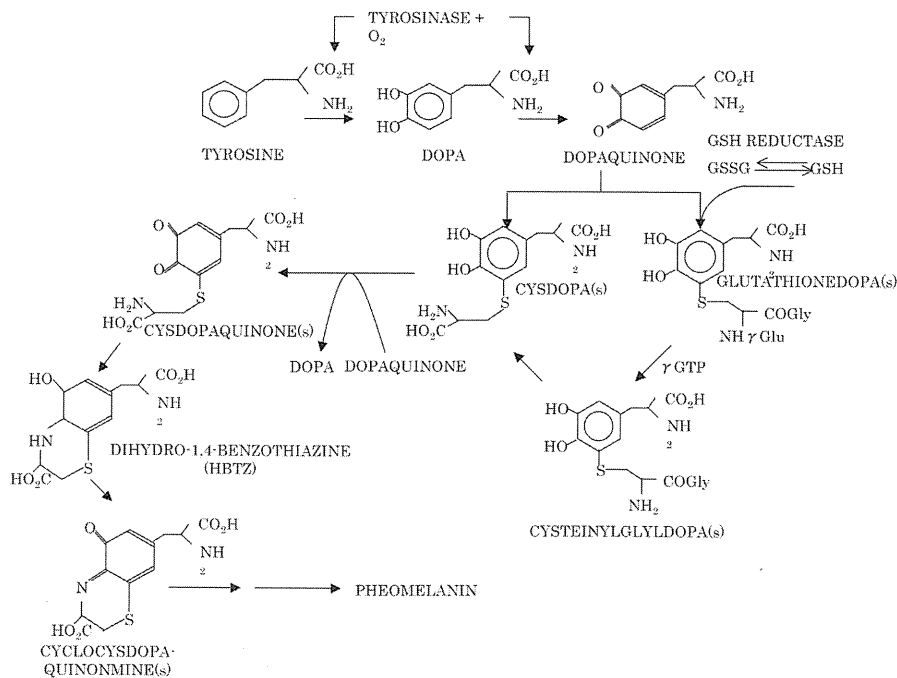


Figure 2 : Chemical reactions of pheomelanin biosynthesis. Metabolic and chemical reactions of pheomelanin synthesis

Table II : Genetic association of MC1R variants and phenotypes in pheomelaninic red hair and skin cancers

MC1R Variant	Frequency in Austrians (%)	Red Hair		SCC/ BCC		MM
		Smith R et al. (1998)	Kenned C et al. (2001)	Box NF et al. (2001)	Palmer JS et al. (2000)	Matichar E et al. (2004)
Val60Leu	12.4	NA	OR = 2.3	OR = 1.4	OR = 1.3	p < 0.0001 OR = 3.99
Asp84Glu	1.1	—	OR = 5.1		OR = 0.8	p = 0.41 OR = 2.16
Val92Met	9.7	NA	NA	OR = 2.1		
Arg142His	0.9		OR = 49.2			p = 0.07
Arg151Cys	11.1	p = 0.0015	OR = 20.7	OR = 2.6	OR = 2.1	p = 0.0002 OR = 6.48
Arg160Trp	7.1	p < 0.001	OR = 12.5	OR = 3.4	OR = 1.9	p = 0.0013 OR = 4.73
Arg163Gln	5.0	—	NA	OR = 1.7		
Asp294His	2.8	p < 0.005	OR = 15.0	OR = 15.0	OR = 2.3	p = 0.058 OR = 3.84

SCC ; Squamous cell carcinoma, BCC ; Basal cell carcinoma, NA ; No association

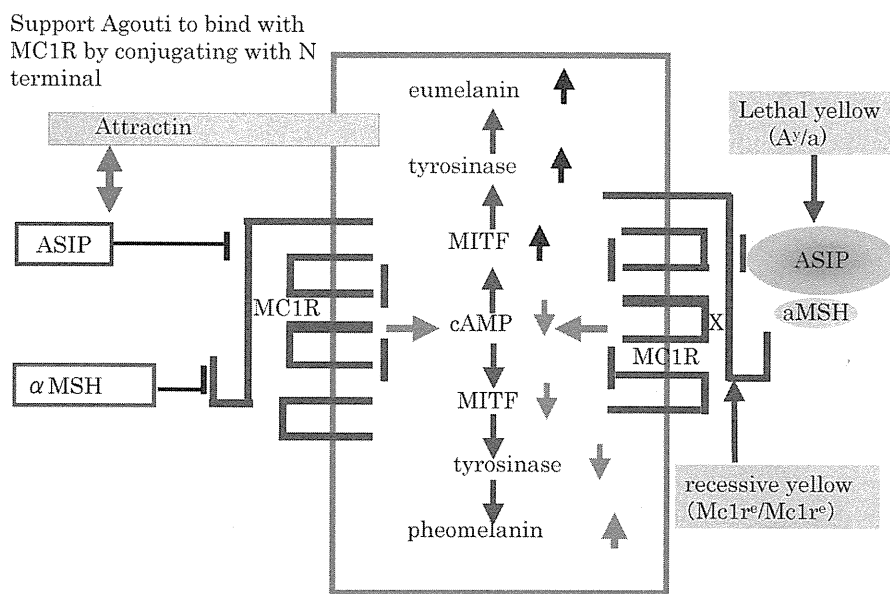


Figure 3 : The interaction of ASIP and α MSH for biosynthesis of eumelanin and pheomelanin

eumelanogenic signal, down-regulating melanogenic enzymes and leading pheomelanin synthesis (Fig. 3)³⁹. Polymorphisms within the MC1R gene are largely responsible for the wide range of skin and hair color among different ethnic groups¹⁵. At least 68 MC1R variants have been identified in humans, and several of them in fair-skinned subjects with red hair are highly associated with skin cancers and melanoma

(Table II)⁴¹.

Eumelanin biosynthesis in EMU and aging of skin

1. Melanogenesis and oxidative stress in EMU after exposure to UVR

Alterations of the EMU in response to exposure to intrinsic and extrinsic factors are often linked to oxidative stress that produces

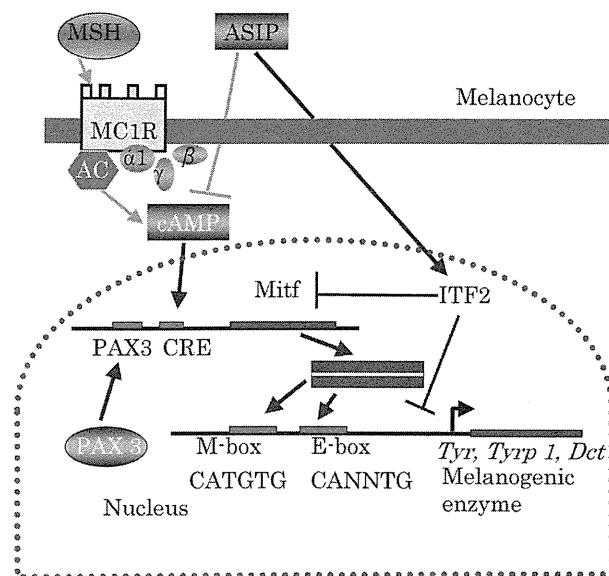


Figure 4 : Involvement of ITF 2 in transcriptional regulation for switch between eumelanin and pheomelanin biosynthesis. The figure was modified from Furumura M et al (Furumura M et al, J Biol Chem 276 : 28147-28154, 2001)

1. ITF 2; ubiquitous basic helix-loop-helix transcription factor (bHLP)
2. Up-regulation of ITF 2 suppresses melanogenic gene expression and Mitf expression
3. Agouti signal protein (ASIP) antagonizes the binding of MSH to MC 1 R

imbalanced redox status beyond the protective capacities of detoxifying enzymes. Melanocytes can produce such cytotoxic products during biosynthesis of melanin pigments in the presence of tyrosinase. This melanin biosynthesis process involves a series of reactions consisting of one-electron transfer system from electron donors (phenol/ catechol amines) to electron acceptors (quinones/ quinone amines), therefore the whole process resolves for the production of "free radicals". Quinones can be toxic at least by two mechanisms, i.e., either directly reacting with the-SH group of essential cellular molecules, or creating oxidative stress by redox cycling which results in superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide. Quinones undergo one-electron reduction by cellular redox system to semiquinones, which are then re-oxidized by O_2 to quinone and $O_2^{\cdot-}$. Semiquinone radicals can also be produced in the melanogenic pathway non-enzymically, through mechanisms involving: a) disproportion of quinone and hydroquinone forms of reactants (catechols and hydroxyindoles); b) oxidation of catechol(amine)s by superoxide; and c) metal ion (iron, copper) -catalyzed oxidation of catechol (amine)s by oxygen (Fig. 5). In addition, UV light/or

physical injury can stimulate semiquinone formation from melanogenic compounds via direct interaction (causing photo-ionization and/or photo-homolysis of phenolic OH groups in catechol amines and hydroxyindoles), or indirectly, through photosensitization (e.g., as in UV-B/ psoralen and UVA [PUVA]). These processes are partly responsible for the UV light-stimulated cytotoxicity⁴⁵.

2. Tyrosinase and LAMP gene families and oxidative stress

Besides tyrosinase, as discussed briefly in the above section, two molecules are related to tyrosinase and are referred to as tyrosinase-related proteins (TYRPs in humans and Tyrps in animals) ; (a) TYRP 1, which is relevant to brown locus protein in mice and (b) TYRP 2, which is also present in melanosomes and has a dopachrome tautomerase (DCT) activity. These three enzymes are called tyrosinase gene family because of the structural homology among them and the identification of respective genes in the same melanocyte cDNA expression library by anti-tyrosinase antibody immunoscreening. Melanosomes and lysosomes share many common structural similarities. We and

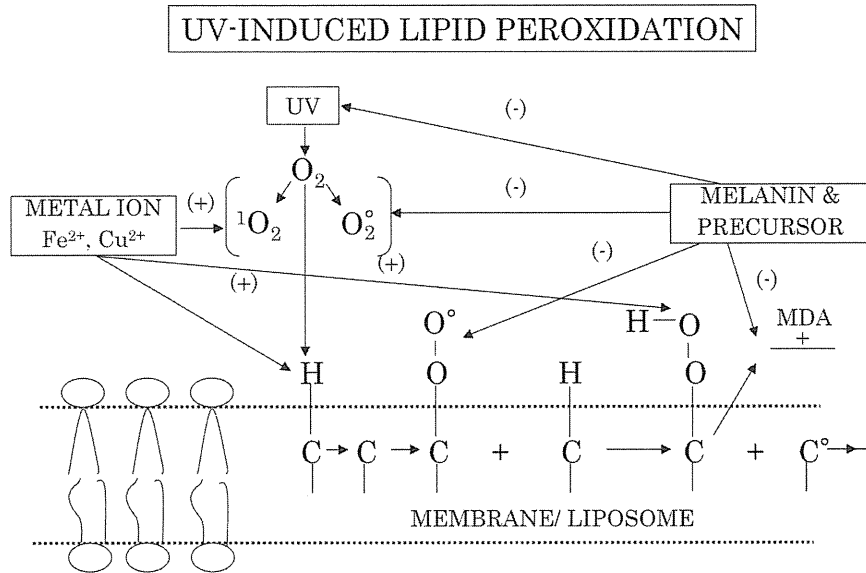


Figure 5 : UV-induced cell damage by lipid peroxidation of the cell membrane

UV-induced cell damage by lipid peroxidation of the cell membrane. Melanin pigments and their precursors, in particular, pheomelanin and its precursor intermediate, 5-S-cysteinyl-dopa can become pro-oxidant causing lipid peroxidation in the presence of metal ions, e.g. Fe^{2+} , Fe^{3+} and Cu^{2+} after exposure to UVR.

other research groups have identified lysosome-associated membrane protein (LAMPs) which are associated with the membrane of the two granules^{27),36),43)}.

They may derive from the common primordial melanogenesis-associated gene. Repeated exposure of human melanocytes to UVB can directly stimulate the expression of tyrosinase and LAMP gene families. Importantly, tyrosinase and TYRP 1 may coordinate together to up-regulate LAMP 1 molecule which is likely to scavenge some of the toxic species generated during melanin metabolism (hydroxyl radicals) through high content of both N- and O-linked oligosaccharides by continuously coating the inner surface of melanosomal membrane^{26),31),43)}.

3. Function and mutation of TYRP genes

Mutations that affect different stages of melanocyte development have been best characterized in experimental mice. The isolation and sequencing of the culprit genes by molecular biology techniques have allowed the determination of the basis of these mutations. The albino mutation of mouse tyrosinase and the brown mutation of Tyrp 1 both alter a cysteine in the EGF motif to serine in albino and different cysteine to tyrosine in brown. Proof of the mutation

status of the alteration in both cases has been provided by analysis of revertants which restore wild type function. In addition, comparison of the phenotypes with those of deletion of the loci indicates that both mutations have complete loss of function of the gene product. On the other hand, DCT (Typr 2) containing the slaty mutation, which has arginine to glutamine change at the first copper-binding site may still have considerable DCT activity.

The function of TYRP 1 is still not well characterized. However, it is believed to be involved in eumelanin synthesis since the brown mutation only affects the eumelanin animals. Jackson et al. demonstrated gene changes in Tyrp 1 mRNA expression and in Tyrp 1 gene structure in certain mouse brown b-locus mutants²²⁾. These findings have provided additional evidence of allelism and allowed an explanation of the phenotypes. The human homologue of TYRP 1 of the mouse brown gene maps to the short arm of chromosome 9 and extends the known region of homology with mouse chromosome 4.

Both recessive and dominant mutations of Tyrp 1 gene have been examined. Some recessive mutations involve single amino acid substitutions, changing an arginine residue to the single sequence cysteine, whereas others affect the

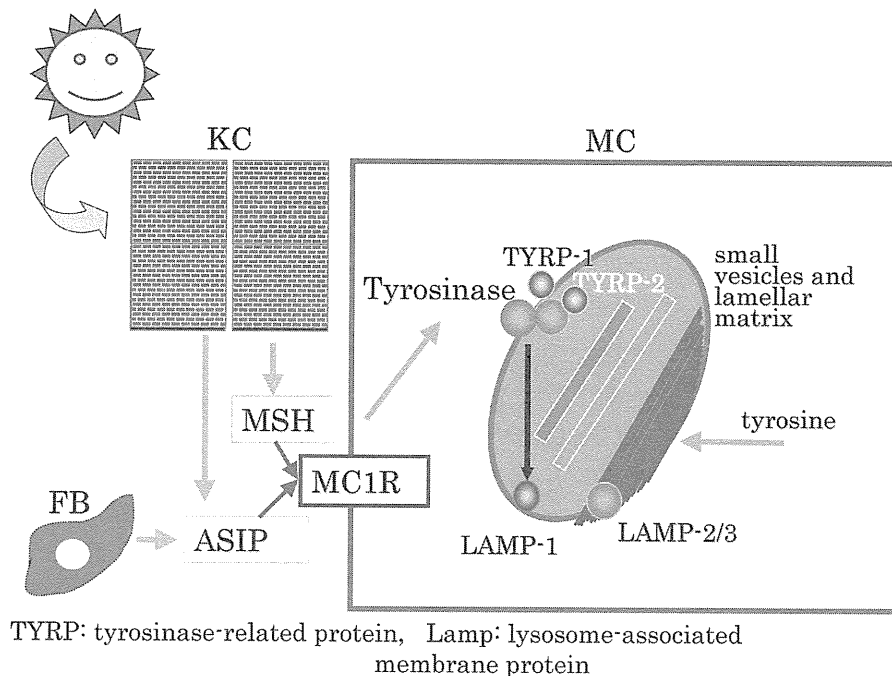


Figure 6 : Coordinated expression of tyrosinase and LAMP gene family proteins in melanogenesis cascade

Pheomelanin biogenesis occurs predominantly in red/blond hair follicles. It is not yet clarified what signals and how the epidermal melanocytes carry out pheomelanin biosynthesis in the skin of these red/blond haired subjects, though pheomelanin is produced together with eumelanin by the same melanocyte in the skin. There will be an increased coordinated upregulation in the expression of TYRP and LAMP gene family proteins after UV exposure.

levels of Tyrp 1 mRNA present in the melanocytes. These different mutations result in either complete loss of function, partial loss of function or temperature sensitive function. Dominant mutations affecting the Tyrp 1 gene have also arisen as a result of a base pair mutation. This type of mutation may destabilize the melanosomal membrane and allow the intermediates of melanogenesis, which are normally sequestered in the melanosomal compartment, to interfere with normal melanocyte function. Another type of dominant mutation may arise due to the Tyrp 1 gene having undergone some re-arrangement, as a result Tyrp 1 is not properly transcribed, leading to melanocyte dysfunction and death. It has been therefore suggested that mutant TYRP 1 protein is involved in toxicity of melanocytes which is associated with inherently toxic melanogenesis process^{28),31)}.

4. Function and mutation of LAMP genes

The involvement of lysosomal constituents including lysosomal enzymes in melanogenesis has long been implicated. As early as 1968,

Novikoff observed the identical distribution of tyrosinase (dopa oxidase) and lysosomal acid phosphatase in the GERL (Golgi-endoplasmic reticulum-lysosome) areas under electron microscopy and proposed that melanosomes have the same developmental sequences as lysosomal granules do. The compartment of both melanosomes and lysosomes is acidic. LAMP 1 and probably LAMP 2 are "household genes" located in lysosomes, constituting a major portion of lysosome-associated membrane proteins. LAMP 1 and LAMP 2 have similar structures and masses (up to 90-120 kDa). The deduced amino acid sequences that a major portion of LAMP 1 and LAMP 2 molecules is exposed continuously in the lumina of lysosomes, and that this protein is extensively glycosylated by both N- and O-glycans. This luminal domain is followed by single membrane-spanning domain and a short-cytoplasmic tail. This structural organization is well conserved among molecules of different species, as well as between LAMP 1 and LAMP 2. The molecules of LAMP 1 and LAMP 2 have been suggested, because of their abundance in

lysosomes, to form a nearly continuous coat on the inner surface of the lysosomal membrane which serves as a barrier to soluble lysosomal hydrolases. Heavy glycosylation, including bulky poly-N-acetyl lactosamines (18 N-linked oligosaccharides), probably plays an important role in protecting LAMP molecules from the cytotoxic action of hydrolases (Fig. 6).

Comparison of LAMP 1 and LAMP 2 amino acid sequences revealed that they are encoded by separate genes located on different chromosomes and most likely produced by the duplication of a primordial gene, which took place early in evolution. Thus, LAMP 1 and LAMP 2 could have distinct functions. According to this view, the human LAMP 1 gene is located at chromosome 13 q 34, whereas the human LAMP 2 gene is located at chromosome Xq 24-25. The human LAMP 1 gene is characterized by having a long intron 4, whereas the exons V-IX are present as a cluster. In contrast, the human LAMP 2 gene is characteristic in having a very long intron 8, and exons IV-V III are present as a cluster. It was shown that LAMP 1 is constitutively synthesized, whereas the synthesis of LAMP 2 is increased during the differentiation of certain embryonic or carcinoma cells. At the present time, we do not know how the mutation of LAMP gene family affects the melanogenesis, although they should have significant influence and have been implicated clinically in abnormal pigmentation seen in such diseases as hypomelanotic macules of vitiligo and café-au-lait macules of Von Recklinghausen's disease³²⁾.

Pheomelanin biosynthesis in EMU and aging of skin

1. Assay of melanin and high content of pheomelanin in melanoma and its precursors

A simple, rapid and sensitive method for the quantitative analysis of eu- and pheomelanin in tissues which makes the isolation of melanin unnecessary^{18),20)}. The method is based on the formation of pyrrole-2, 3,5-tricarboxylic acid (PTCA) from eumelanin by permanganate oxidation, and aminohydroxyphenylalanine isomers (AHP) from pheomelanin by hydriodic acid hydrolysis. The products are separated and calculated by high-pressure liquid chromatography (HPLC).

The HPLC analysis of melanin has been successfully applied to examine eu- and phe-

nomelanin in the *in vivo* and *in vitro* cells and tissues^{18),21)}, human hair²³⁾, mouse hair^{8),19)}, and cultured melanocytes.⁴⁷⁾ The content of dopa and 5-S-cysteinyl-dopa in both the culture medium and the cells has been used as a marker of differentiation of melanoma cells^{2),3)}.

Our previous electron microscopic studies⁵¹⁾ showed that melanocytes of dysplastic melanocytic nevi (DMN) were rich in abnormal melanosomes which exhibited features consistent with pheomelanosomes. A significantly high content of pheomelanin was found in DMN lesions, indicating that these abnormal melanosomes may be pheomelanin⁴⁶⁾. UV sensitivity in fair-skinned subjects at high risk for melanoma was found to be associated with high pheomelanin and low eumelanin levels⁵²⁾. Epidemiologic data revealing that fair-skinned individuals are more susceptible to skin cancers^{4),11)} are linked to greater phototoxicity of pheomelanin (Table II)⁵³⁾. In contrast, an examination of eumelanin and pheomelanin concentrations in human skin before and after exposure to UVR showed that factors other than the amount of pheomelanin may be important in determining cancer susceptibility¹⁶⁾. Ye et al. found that different molecular constituents in pheomelanin are responsible for emission, transient absorption and oxygen photo-consumption, indicating the necessity of combining results from many techniques when trying to develop a unified view of pigment reactivity and electronic structure⁵³⁾. It would be important to examine the type of melanogenesis with respect to the degree of skin damage and abnormal pigmentation in the photo-aged skin.

2. UV photoproducts of melanin pigmentation and cytotoxicity

Relatively pure eumelanin and pheomelanin have been isolated and, when subjected to UVR, behave quite differently Menon IA il³⁸⁾. investigated the possibility that UVR in the presence of pheomelanin may be more harmful to cells than UVR in the presence of eumelanin. It appears that the production of a short-lived singlet oxygen and its conversion to hydroxyl radicals by the photo-oxidation of pheomelanin are well established^{40),54)}. The effects of UVR and visible light upon Ehrlich ascites carcinoma cells in the presence of the melanin isolated from human black hair (eumelanin) or from red hair (pheomelanin) were investigated. UV irradiation of these cells was found to produce cell ly-

sis. The effects were more pronounced when UVR was done in the presence of pheomelanin from red hair than in the presence of black hair eumelanin. Irradiation of the cells in the presence of red hair pheomelanin also decreased the transplantability of these cells. Ehrlich ascites carcinoma cells were injected into mice intraperitoneally after UV irradiation in the presence of eumelanin or pheomelanin. The ability of such treated cells to grow was assessed by changes in animal weight. It was found that carcinoma cells subjected to UVR in the presence of pheomelanin showed impaired growth, which was not seen with UVR alone or UVR in the presence of eumelanin.

Another recent study⁴⁴ showed that, when treated with UVA radiation, human red hair melanin was considerably more damaging to Ehrlich ascites cells than eumelanin from black hair. Peritoneal rat mast cells, incubated with pheomelanin and irradiated with UVR, released histamines without cell lysis, whereas such changes did not occur in the presence of eumelanin. These findings may have clearly shown the different biological effect of eumelanin and pheomelanin. It is also important to note that the precursors of these two melanin pigments behave dual role of phototoxic and photoprotective agents⁴⁸.

3. UV-photoproducts of pheomelanin in aging process

Electron-spin resonance spectroscopy ex-

periments by Sealy et al⁵⁰. on black and red melanins, suggested that red melanin (pheomelanin) contained a specific kind of free radical (s) not present in black eumelanin¹¹, finding that pheomelanin was photo-destroyed, in the presence of oxygen, by UVR. Harsanyi et al.,¹⁶ using the reversion test of Ames, demonstrated that pheomelanin became mutagenic after exposure to UVR³⁴, describing photo-initiated DNA damage by melanogenic intermediates of 5-S-cysteinyl-dopa origin. The binding of these molecules to DNA was activated by 300 nm UVR and resulted in single-strand breaks.

Catalase structure and activity was seriously affected by photo-oxidation of its own substrates, hydrogen peroxide, owing to cleavage of its porphyrin active site. Recently the over-expression of mitochondrial catalase in the murine model increased the lifespan of the mice by 40%, indicating the importance of this enzyme in the aging process⁴⁹. Importantly UVA-irradiated pheomelanin altered the structure of catalase and decrease its activity in human skin³⁷. It would be interesting to see to what extent UV-photoproducts of pheomelanin are involved in aging process of the skin. It is likely that they will be significantly involved in Caucasians with light-skin and blue eyes, having significant alterations of MC1R and pheomelanin in skin and hair, and high risk for developing melanoma (Fig. 7) .

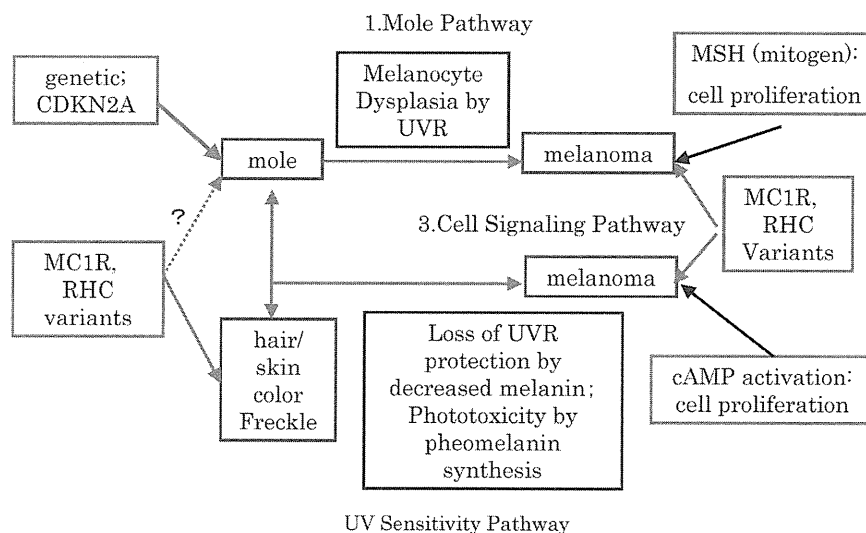


Figure 7 : Involvement of Red hair phenotype and pheomelanin biosynthesis in melanoma development. The figure was modified from Sturn RA (Sturn RA, Melanoma Res 12 : 405-16, 2002).

Summary and conclusion

There are two forms of melanin pigments, i. e., eu- and pheomelanin in the skin. The major determinant of skin protection from external stimuli such as UVR is the total amount and distribution pattern of eumelanin in the EMU. While biosynthesis process and biological role of eumelanin after exposure to UVR are well characterized, the nature and photo-biological role of pheomelanin in the EMU is little known. Based upon previous publications including ours, it seems clear that pheomelanin and its precursor cysteinyl-dopas are photochemically unstable in the presence of UVR. Free radicals are produced and photolysis of pheomelanin and its precursor pigments may lead to severe DNA damage. The extent of cell damage and lysis is likely much less in young skin as compared to the aged one. No one, however, does not appear to have investigated these phenomena directly on melanocytes during aging process, nor has anyone looked at alterations in the type of melanogenesis in aging process of the skin.

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Reference

- 1) Aubert C, Rosengren E, Rorsman H, Rougé F: Differentiation of melanocytes in cultures of primary malignant melanoma indicated by 5-S-cysteinyl-dopa formation. *J Natl Cancer Inst* 55: 1237-1328, 1975.
- 2) Aubert C, Rougé F, Galindo JR: Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation in vitro. *J Natl Cancer Inst* 72: 3-12, 1984.
- 3) Barsh GS: Regulation of pigment type switching by agouti, melanocortin signaling, attractin and mahoganoid. In: *The Pigmentary System, Second Edition* ed by Nordlund JJ, Boissy RE, Hearing VJ, King RA, Oetting WS, and Ortonne JP, Blackwell Publishing, Massachusetts 395-409, 2006.
- 4) Bastiaens MT, ter Huurne JA, Kielich C, Gruis NA, Westendorp RG, Vermeer BJ, Bavinck JN: Leiden Skin Cancer Study Team. Melanocortin-1 receptor gene variants determine the risk of nonmelanoma skin cancer independently of fair skin and red hair. *Am J Hum Genet* 68: 884-94, 2001.
- 5) Bennett D: Mechanisms of differentiation in melanoma cells and melanocytes. *Environ Health Perspect* 80: 49-59, 1989.
- 6) Bennett D, Lamoreux ML: The color loci of mice—a genetic century. *Pigment Cell Res* 16: 333-44, 2003.
- 7) Bertolotto C, Abbe P, Hemesath TJ, Bille K, Fisher DE, Ortonne JP, Ballotti R: Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. *J Cell Biol* 142: 827-35, 1998.
- 8) Burchill SA, Thody AJ, Ito S: Melanocyte-stimulating hormone, tyrosinase activity and the regulation of eumelanogenesis and pheomelanogenesis in the hair follicular-melanocytes of the mouse. *J Endocr* 109: 15-21, 1986.
- 9) Chedekel MR, Post PW, Deibel RM, Kalus M: Photodestruction of pheomelanin. *Photochem Photobiol* 2: 651, 1977.
- 10) Cui R, Widlund HR, Feige E, Lin JY, Wilensky DL, Igras VE, D'Orazio J, Fung CY, Schanbacher CF, Granter SR, Fisher DE: Central role of p53 in the suntan response and pathologic hyperpigmentation. *Cell* 128: 853-64, 2007.
- 11) Davies TW, Treasure FP, Welch AA, Day NE: Diet and basal cell skin cancer: results from the EPIC-Norfolk cohort. *Br J Dermatol* 146: 1017-1022, 2002.
- 12) García-Borrón JC, Sánchez-Laorden BL, Jiménez-Cervantes C: Melanocortin-1 receptor structure and functional regulation. *Pigment Cell Res* 18: 393-410, 2005.
- 13) Gilchrist BA, Zhai S, Eller MS, Yarosh DB, Yaar M: Treatment of human melanocytes and S 91 melanoma cells with the DNA repair enzyme T4 endonuclease V enhances melanogenesis after ultraviolet irradiation. *J Invest Dermatol* 101: 666-672, 1993.
- 14) Gilchrist BA, Park HY, Eller MS, Yaar M: Mechanisms of ultraviolet light-induced pigmentation. *Photochem Photobiol* 63: 1-10, 1996.
- 15) Ha T, Naysmith L, Waterston K, Oh C, Weller R, Rees JL: Defining the quantitative contribution of the melanocortin 1 receptor (MC 1 R) to variation in pigmentary phenotype. *Ann N Y Acad Sci* 994: 339-347, 2003.
- 16) Harsanyi ZP, Post PW, Brinkmann JP, Chedekel MR, Deibel RM: Mutagenicity of

- melanin from human red hair. *Experientia* 36 : 291-292, 1980.
- 17) Hennessy A, Oh C, Diffey B, Wakamatsu K, Ito S, Rees J: Eumelanin and pheomelanin concentrations in human epidermis before and after UVB irradiation. *Pigment Cell Res* 18 : 220-223, 2005.
 - 18) Ito S, Jimbow K: Quantitative analysis of eumelanin and pheomelanin in hair and melanomas. *J Invest Dermatol* 80 : 268-272, 1983.
 - 19) Ito S, Fujita K, Takahashi H, Jimbow K: Characterization of melanogenesis in mouse and guinea pig hair by chemical analysis of melanins and of free and bound dopa and 5-S-cysteinyl-dopa. *J Invest Dermatol* 83 : 12-14, 1984.
 - 20) Ito S, Fujita K: Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. *Anal Biochem* 144 : 527-536, 1985.
 - 21) Ito S, Wakamatsu K: Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. *Pigment Cell Res* 16 : 523-531, 2003.
 - 22) Jackson IJ, Chambers DM, Budd PS, Johnson R: The tyrosinase-related protein-1 gene has a structure and promoter sequence very different from tyrosinase. *Nucleic Acids* 19 : 3799-3804, 1991.
 - 23) Jimbow K, Ishida O, Ito S, Hori Y, Witkop CJ Jr, King RA. Combined chemical and electron microscopic studies of pheomelanosomes in human red hair. *J Invest Dermatol* 81 : 506-511, 1983.
 - 24) Jimbow K, Fitzpatrick TB, Quevedo WC Jr: Formation, chemical compositions and functions of melanin pigments in mammals. In: *Biology of the Integument* ed by AG Matoltsy, Springer-Verlag, Berlin 2 : 278-296, 1986.
 - 25) Jimbow K, Fitzpatrick TB, Wick M: Biochemistry and physiology of melanin pigmentation. In: *Physiology and Molecular Biology of the Skin, Vol. II* ed by LAGoldsmith, Oxford University Press, New York 873-909, 1991.
 - 26) Jimbow K, Hara H, Vinayagamoorthy T, Luo D, Dakour J, Yamada K, Dixon W, Chen H: Molecular control of melanogenesis: Functional assessment of tyrosinase and Lamp gene families by UV exposure and gene co-transfection, and cloning of a cDNA encoding calnexin, a possible melanogenesis "chaperone". *J Dermatol* 21 : 894-906, 1994.
 - 27) Jimbow K, Luo D, Chen H, Hara H, Lee MH: Co-ordinated mRNA and protein expression of human Lamp-1 in induction of melanogenesis after UV-B exposure and co-transfection of human tyrosinase and TRP-1 cDNAs. *Pigment Cell Res* 7 : 311-319, 1994.
 - 28) Jimbow k, Gomez PF, Toyofuku K, Chang D, Miura S, Tsujiya H, Park JS: 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 Res* 10 : 206-213, 1997.
 - 29) Jimbow K, Prota G, Quevedo WC: *Biology of Melanocytes*. In: *Fitzpatrick's Dermatology in General Medicine, Fifth Edition* ed by Freedberg IM, Eisen AZ, Wolff E, Austen KF, Goldsmith LA, Katz SI, Fitzpatrick TB, McGraw-Hill, New York 192-220, 1998.
 - 30) Jimbow K, Chen H, Gomez PF, Hirosaki K, Shinoda K, Salopek TG, Matsusaka H, Jin HY, Yamashita T: Intracellular vesicular trafficking of tyrosinase gene family protein in eu- and pheomelanosome biogenesis. *Pigment Cell Res* 13 (suppl) : 110-117, 2000.
 - 31) Jimbow K, Park JS, Kato F, Hirosaki K, Toyofuku K, Hua C, Yamashita T: Assembly, target signal and intracellular transport of tyrosinase gene family proteins in the initial stage of melanosome biogenesis. *Pigment Cell Res* 13 : 222-229, 2000.
 - 32) Jimbow K, Chen H, Park JS, Thomas PD: Increased sensitivity of melanocytes to oxidative stress and abnormal expression of tyrosinase related protein in vitiligo. *Br J Dermatol* 144 : 55-65, 2001.
 - 33) Khlgtian MK, Hadshiew IM, Asawanonda P, Yaar M, Eller MS, Fujita M, Norris DA, Gilchrist BA: Tyrosinase gene expression is regulated by p 53. *J Invest Dermatol* 118 : 126-32, 2002.
 - 34) Koch WH, Chedekel MR: Photoinitiated DNA damage by melanogenic intermediates in vitro. *Photochem Photobiol* 44 : 703, 1986.
 - 35) Levy C, Khaled M, Fisher DE: MITF: master regulator of melanocyte development and melanoma oncogene. *Trends Mol Med* 12 : 406-14, 2006.
 - 36) Luo D, Chen H, Jimbow K: Co-transfection

- of genes encoding human tyrosinase and tyrosinase-related protein-1 prevents melanocyte death and enhances melanin pigmentation and gene expression of Lamp-1. *Exp Cell Res* 213: 231-241, 1994.
- 37) Maresca V, Flori E, Briganti S, Camera E, Cario-André M, Taïeb A, Picardo M: UVA-induced modification of catalase charge properties in the epidermis is correlated with the skin phototype. *J Invest Dermatol* 126: 182-90, 2006.
- 38) Menon IA, Persad S, Ranadive NS, Haberman HF: Effects of ultraviolet-visible irradiation in the presence of melanin isolated from human black or red hair upon Ehrlich ascites carcinoma cells. *Cancer Res* 43: 3165-3169, 1983.
- 39) Millar SE, Miller MW, Stevens ME, Barsh GS: Expression and transgenic studies of the mouse agouti gene provide insight into the mechanisms by which mammalian coat color patterns are generated. *Development* 121: 3223-3232, 1995.
- 40) Nordberg J, Arnér ES: Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radic Biol Med* 31: 1287-312, 2001.
- 41) Park HY, Kosmadaki M, Yaar M, Gilchrist BA: Cellular mechanisms regulation human melanogenesis. *Cell Mol Life Sci*, 2009 (Epub ahead of print).
- 42) Parrish JA, Jaenicke KF, Anderson RR: Erythema and melanogenesis action spectra of normal human skin. *Photochem Photobiol* 36: 187-91, 1982.
- 43) Rad H, Yamashita T, Jin HY, Hirosaki K, Wakamatsu K, Ito S, Jimbow K: Tyrosinase-related proteins suppress tyrosinase-mediated cell death of melanocytes and melanoma cells. *Exp Cell Res* 298: 317-328, 2004.
- 44) Ranadive NS, Shirwadkar S, Persad S, Menon IA: Effects of melanin-induced free radicals on the isolated rat peritoneal mast cells. *J Invest Dermatol* 86: 303-307, 1986.
- 45) Reszka K, Jimbow K: Electron-donor and acceptor properties of melanin pigments in the skin. In: *Oxidative Stress In Dermatology* ed by J Fuchs and L Packer, Marcel Dekker Inc, New York 287-320, 1993.
- 46) Salopek TG, Yamada K, Ito S, Jimbow K: Dysplastic melanocytic nevi contain high levels of pheomelanin: quantitative comparison of pheomelanin/eumelanin levels between normal skin, common nevi, and dysplastic nevi. *Pigment Cell Res* 4: 172-9, 1991.
- 47) Sato C, Ito S, Takeuchi T: Enhancement of pheomelanogenesis by L-dopa in the mouse melanocyte cell line, TM 10, in vitro. *J Cell Sci* 87: 507-12, 1987.
- 48) Schmitz S, Thomas PD, Allen TM, Poznansky MJ, Jimbow K: Dual role of melanins and melanin precursors as photoprotective and phototoxic agents: inhibition of ultraviolet radiation-induced lipid peroxidation. *Photochem Photobiol* 61: 650-655, 1995.
- 49) Schriener SE, Linford NJ, Martin GM, Treuting P, Ogburn CE, Emond M, Coskun PE, Ladiges W, Wolf N, Van Remmen H, Wallace DC, Rabinovitch PS: Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* 308: 1909-11, 2005.
- 50) Sealy RC, Hyde JS, Felix CC, Menon IA, Prota G: Eumelanins and pheomelanins: characterization by electron spin resonance spectroscopy. *Science* 217: 545-7, 1982.
- 51) Takahashi H, Horikoshi T, Jimbow K: Fine structural characterization of melanosomes in dysplastic nevi. *Cancer* 56: 111-23, 1985.
- 52) Vincenzi MR, d'Ischia M, Napolitano A, Procaccini EM, Riccio G, Monfrecola G, Santoianni P, Prota G: Pheomelanin versus eumelanin as a chemical indicator of ultraviolet sensitivity in fair-skinned subjects at high risk for melanoma: a pilot study. *Melanoma Res* 8: 53-58, 1998.
- 53) Wenczl E, Van der Schans G, Roza L, M Kolb R, Timmerman AJ, Smit N, Pavel S, Schothorst A: (Pheo) Melanin Photosensitizes UVA-Induced DNA Damage in Cultured Human Melanocytes. *J Invest Dermatol* 111: 678-682, 1998.
- 54) Wood JM, Schallreuter KU: UVA-irradiated pheomelanin alters the structure of catalase and decreases its activity in human skin. *J Invest Dermatol* 126: 13-14, 2006.
- 55) Ye T, Pawlak A, Sarna T, Simon JD: Different molecular constituents in pheomelanin are responsible for emission, transient absorption and oxygen photoconsumption. *Photochem Photobiol* 84: 437-443, 2008.

Research Article

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

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

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1. Introduction

Melanogenesis is inherently cytotoxic and uniquely occurs in melanocytic cells; thus, tyrosine analogs that are tyrosinase substrates are good candidates for melanoma-specific targeting and therapy [1]. *N*-propionyl and *N*-acetyl derivatives of 4-*S*-cysteaminylphenol (NPr- and NAcCAP) were synthesized and found to possess both cytostatic and cytotoxic effects on *in vivo* and *in vitro* melanomas through the

oxidative stress resulting from production of cytotoxic free radicals [2–6]. We now provide evidence that the unique melanogenesis cascade can be exploited for developing a chemo-thermo-immunologic approach (CTI Therapy) targeted to melanoma by conjugating NPrCAP with magnetite nanoparticles (NPrCAP/M).

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

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

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

2. Materials and Methods

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

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

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

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

2.4. Treatment Protocols. Animal experiments were carried out using the four protocols described below. Each experimental group of Protocols number 1 through number 4 consisted of six to eleven mice. All the treatment protocols were again approved by the Animal Experimental Ethics Committee of Sapporo Medical University. The experimental conditions for melanoma transplantation and the methods of NPrCAP/M administration were identical in all four protocols and all the animal experiments including drug

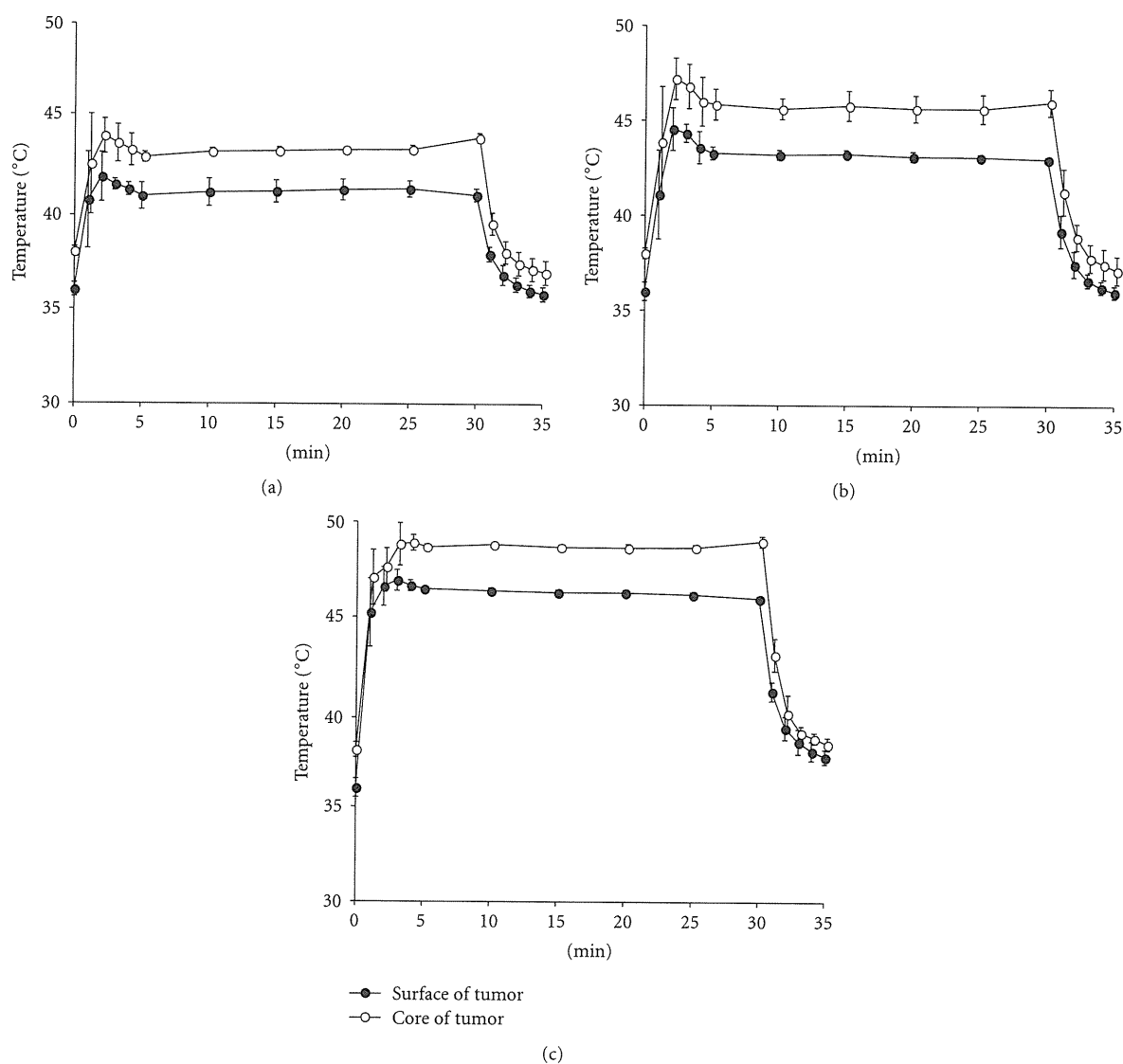


FIGURE 1: Shifts of temperatures at the core and the surface of tumors during AMF exposure. The temperature at the tumor surface was maintained at 41°C (a), 43°C (b), and 46°C (c) for 30 minutes by adjusting the power of the AMF generator. Temperatures of both the tumor surface and core were measured simultaneously.

administration were carried out after anesthesia by diethyl ether.

2.4.1. Protocol Number 1: Effect of NPrCAP/M with and without AMF on Re-Challenge Tumor Growth. On day 5 after the *s.c.* transplantation of B16 melanoma cells, mice were divided into four groups. In Groups I and II mice received *s.c.* administration of 0.1 mL of 40.0 mg/mL aminosilane-coated magnetite (M) once a day every other day for a total of three days (days 6, 8, and 10) with AMF (Group II) or without AMF (Group I). In Groups III and IV mice

received *s.c.* administration of 0.1 mL of NPrCAP/M (4.0 mg magnetite equivalent) once a day every other day for a total of three days (days 6, 8, and 10) with (Group IV) or without (Group III) AMF. The temperature at the tumor surface was maintained at 43°C during exposure for 30 minutes by controlling AMF intensity. Mice of a control group of the same age and sex received *s.c.* transplantation of melanoma cells into the right flank, as with the treated groups. On day 13 after the primary transplantation all mice underwent total resection of melanoma nodules. On day 53 after the first transplantation (postoperative day 40), surviving mice in each group received a second transplantation of B16

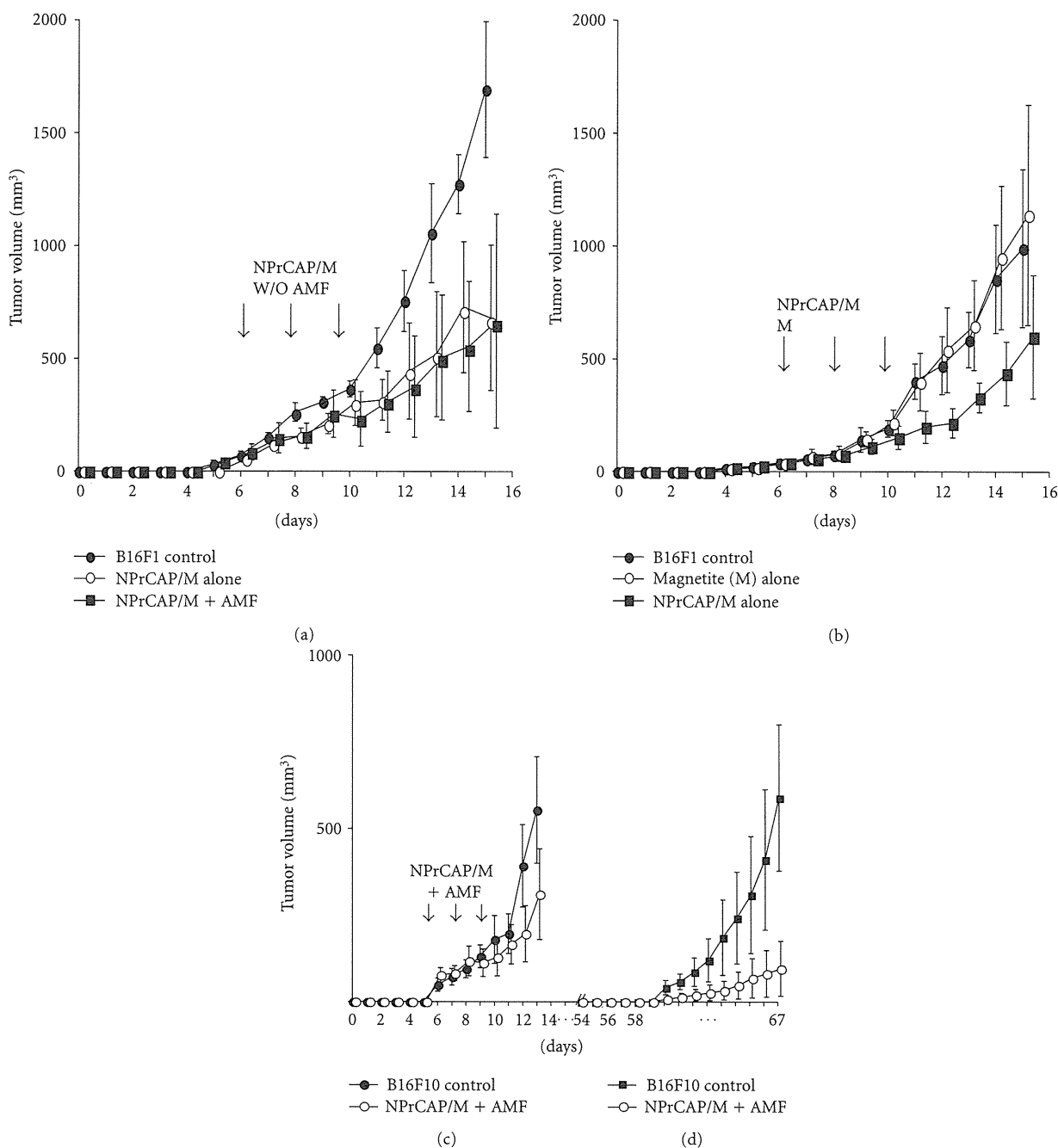


FIGURE 2: Growth curves of primary B16 melanomas. Experimental conditions and statistical analyses of (a), (b), (c) and (d) are identical to those of Protocol number 1. (a) Growth curve of B16F1 melanoma cells treated with NPrCAP/M alone without heat ($n = 10$) and with heat by AMF exposure ($n = 10$) at days 6, 8, and 10. These two groups showed a significant growth inhibition compared to that of control naive mice ($n = 10$) by Dunnett's test ($P < .01$). Importantly, there is no significant difference between the two groups with and without AMF exposure. (b) Growth curve of B16F1 melanoma cells treated with magnetite alone ($n = 10$) and NPrCAP/M alone without AMF exposure ($n = 10$). Mice with magnetite alone did not show any growth inhibition whereas NPrCAP/M alone resulted in a significant growth inhibition of primary melanoma ($P < .01$). (c) Comparison of growth inhibition of B16F10 primary melanomas after treatment with NPrCAP/M with AMF exposure. Compared to control naive mice ($n = 10$) transplanted with B16F10 cells without any treatment, those mice ($n = 10$) with NPrCAP/M plus AMF exposure showed a significant growth inhibition of primary B16F10 melanomas with a similar degree to that of B16F1 melanoma (Figure 2(a)) ($P < .01$ by Dunnett's test). (d) Comparison of the growth inhibition of B16F10 secondary, re-challenge melanoma. The mice ($n = 10$) with NPrCAP/M plus AMF exposure showed a marked growth inhibition compared to control naive mice without any treatment ($n = 10$).