Comparison of biological significance of eu-and pheomelanin pigmentation in skin aging process

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Abstract

This article further discusses, in conjunction to our previous report in Geriatric Dermatology Seminar, vol.4, 2009,[18] some aspects in biological significance of melanin pigmentation to the skin aging process. There are two forms of melanin pigments, i.e., eu- and pheomelanin, in the skin. We discussed here again what regulates for (a) cellular and molecular significance of eumelanin biosynthesis to the skin aging and (b) pheomelanin biosynthesis and its involvement in the skin aging including photo-carcinogenesis. The biological alteration in the epidermal melanin unit (EMU) is one of the major events in the skin aging process. The important determinant of skin protection from external stimuli such as UV radiation (UVR) is the total amount and distribution pattern of eumelanin in the EMU. In contrast, pheomelanin and its precursors 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. Their oxidation products produce short-lived singlet oxygen and its conversion to hydroxyl radials, thus affecting significantly in the skin aging process.

Abbreviation:

AHP, aminohydroxy phenylalanine; APs, adaptor proteins; ASIP, agouti signaling protein; DCT, dopachrome tautomerase; DNM, dysplastic melanocytic nevi; EMU epidermal melanin unit; GERL, Golgi-endoplasmic reticulum-lysosome; EPR, electron paramagnetic resonance; HPLC, high-pressure liquid chromatography; HSP, Hermansky-Pudlak syndrome; LAMP, lysosome-associated membrane protein; Mc 1 r, melanocortin-1 receptor; Mgrn, mahogunin; MITF, microphthalmia-associated transcription factor; αMSH, alfa melanocyte-stimulating hormone; mV vs. NHE, millivolt vs. normal hydrogen electrode; PhO·, phenoxyl radical; POMC, pro-opiomelanocortin; PTCA, Pyrole-2,3,5-tricarboxylic acid; PUVA, psoralen-ultraviolet A; TGN, trans-Golgi network; TYRP (Tyrp), tyrosinase-related proteins; UVA, ultraviolet A; UVB, ultraviolet B; UVC, ultraviolet C; UVR, ultraviolet radiation; DHI 2 CA, dihydroxyndole-2-carboxylic acid; 5, 6 DHI 1 Me, 5,6 dihydroxyindole-1-methyl

Keywords: Melanin pigmentation, Aging process of skin, Eumelanin, Pheomelanin and Epidermal melanin unit.

Melanin pigmentation of human skin color can be photobiologically subdivided into two components. The first, constitutive skin color, designates the amount of cutaneous melanin pigmentation generated in accordance with cellular genetic programs in the absence of direct influences by radiations, usually of solar origin. It is generally taken to be the level of pigmentation in those parts of the body habitually shielded from light. The second, facultative (inducible) skin color or "tan" characterizes the short-lived immediate tanning reaction and absolute increases in melanin pigmentation or delayed tanning above the constitutive level edited by direct exposure of the skin to UV light. Facultative color change (delayed tanning) is considered to be reversible in that the hyperpigmentation of the skin tends to decline over time toward

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the constitutive level when exposure to UVR is discontinued. Skin pigmentation induced by endocrine changes as in pregnancy is another type of facultative color change. In turn, alterations in endocrine balance may significantly influence the response of human skin to UVR. Accordingly, facultative color changes in man arise from the complex interplay of light, hormones and genetic potential of the epidermal melanin unit (EMU), the basic multi-cellular organ of melanin metabolism that affects significantly to the skin aging process.

1. Induction of melanogenesis and oxidative stress after exposure to UV radiation

The EMU is composed of the orderly interaction of a melanocyte and associated pool of keratinocytes with four major biological and biochemical processes, i.e.,(a) the activation of melanocyte and synthesis of melanosomes after exposure to UVR,(b) melanization of these melanosomes within the melanocyte,(c) their transfer from the tip of melanocytic dendrite to surrounding keratinocytes and (d) their degradation within keratinocytes and exfoliation from them. If there is any alteration of these processes, hypo- or hyperpigmentation occurs, resulting in various skin color which ranges from white, light brown, brown to black color (Fig. 1).

Alterations of the EMU in response to exposure to intrinsic and extrinsic factors are often linked to oxidative stress that produces imbalanced redox status beyond the protective capacities of detoxifying enzymes (Fig.2). Melanocytes can produce such cytotoxic products during biosynthesis of melanin pigments. The biosynthetic pathway of melanin pigments is catalyzed by the enzyme tyrosinase. Tyrosinase requires oxygen for its enzymic activity, and it catalyzes two-electron oxidation processes. which consist of one-electron transfer system from electron donors (phenol/ catechol amines) to electron acceptors(quinones/ quinine amines), therefore the whole process resolves for the production of "free radicals".

Phenoxyl radicals (PhO・) are formed during radiolytic oxidation of or tyrosine or phenol.[7,49,50] They are very strong oxidizing agents, as indicated by their redox potential (Table). They may, therefore, oxidize many biological electron donors. Because PhO・ is an one-electron oxidant, the semiquinone radical from hy-

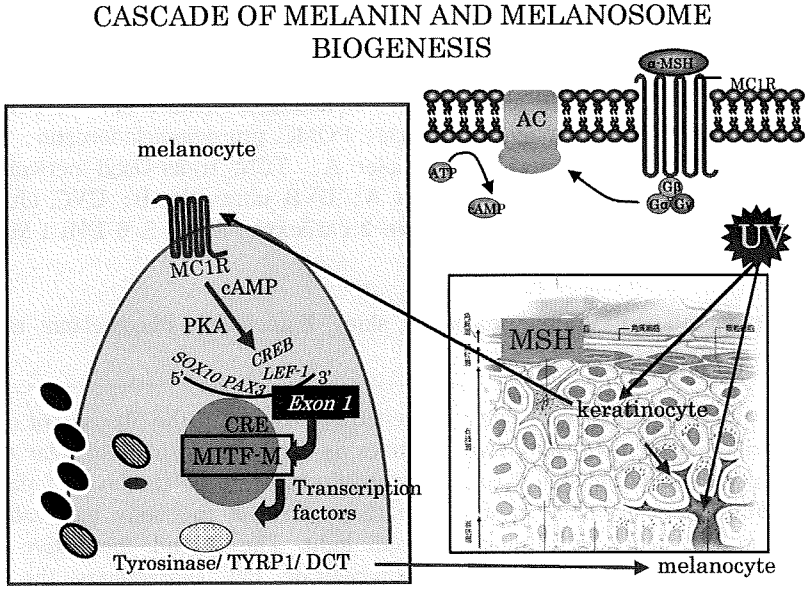

Figure 1：UVR to the skin stimulates the cascade of melanin biosynthesis through activation of αMSH pre-existing in keratinocytes and melanocytes, as well as new synthesis of αMSH in these two cell types. MSH will binds melanocortin 1 receptor (MC 1 R) present on the cell surface of the melanocyte that will then activate microphthalmia associated transforming factor (MITF-M), leading the new synthesis and activation of tyrosinase and its related proteins (tyrosinase related protein 1 : TYRP 1 and dopachrome tautomerase : DCT).

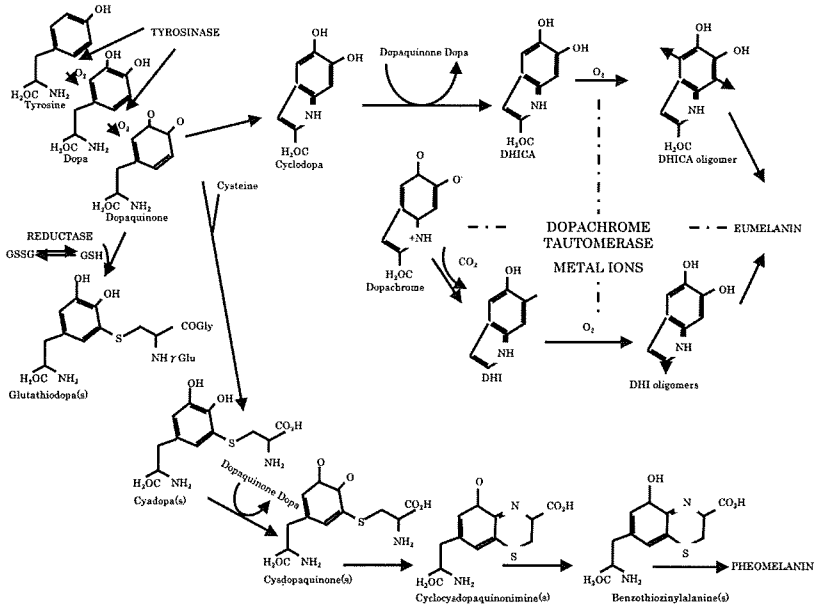

Figure 2: Eumelanin chemical comparison in the biosynthesis and pheomelanin.

One-Electron Redox Potential (m V vs. NHE) of Some Redox Couples at pH 7.4

Redox couple	E_7	Ref.
$Q^{\cdot-}/Q^{H}_{2}$		
Catechol	530	46
*P*Hydroquinone	459	46
DOPA	460	25
6-OH-DOPA	-110(pH 13.5)	47
$Q/Q^{\cdot-}$		
*o*Benzoquinone	210	49
*p*Benzoquinone	99	49
$ArO^{\cdot}$/ ArOH		
Phenol	950	25
Tyrosine	940	6
4-Hydroxyanisole(4-HA)	600	46
5-Hydroxyindole(5HI)	216(pH 13.5)	46
5-Hydroxytryptophan(5HT)	208(pH 13.5)	
$O_2/O_2^{\cdot-}$	-155	49

MV vs. NHE: millivolt vs. normal hydrogen electrode

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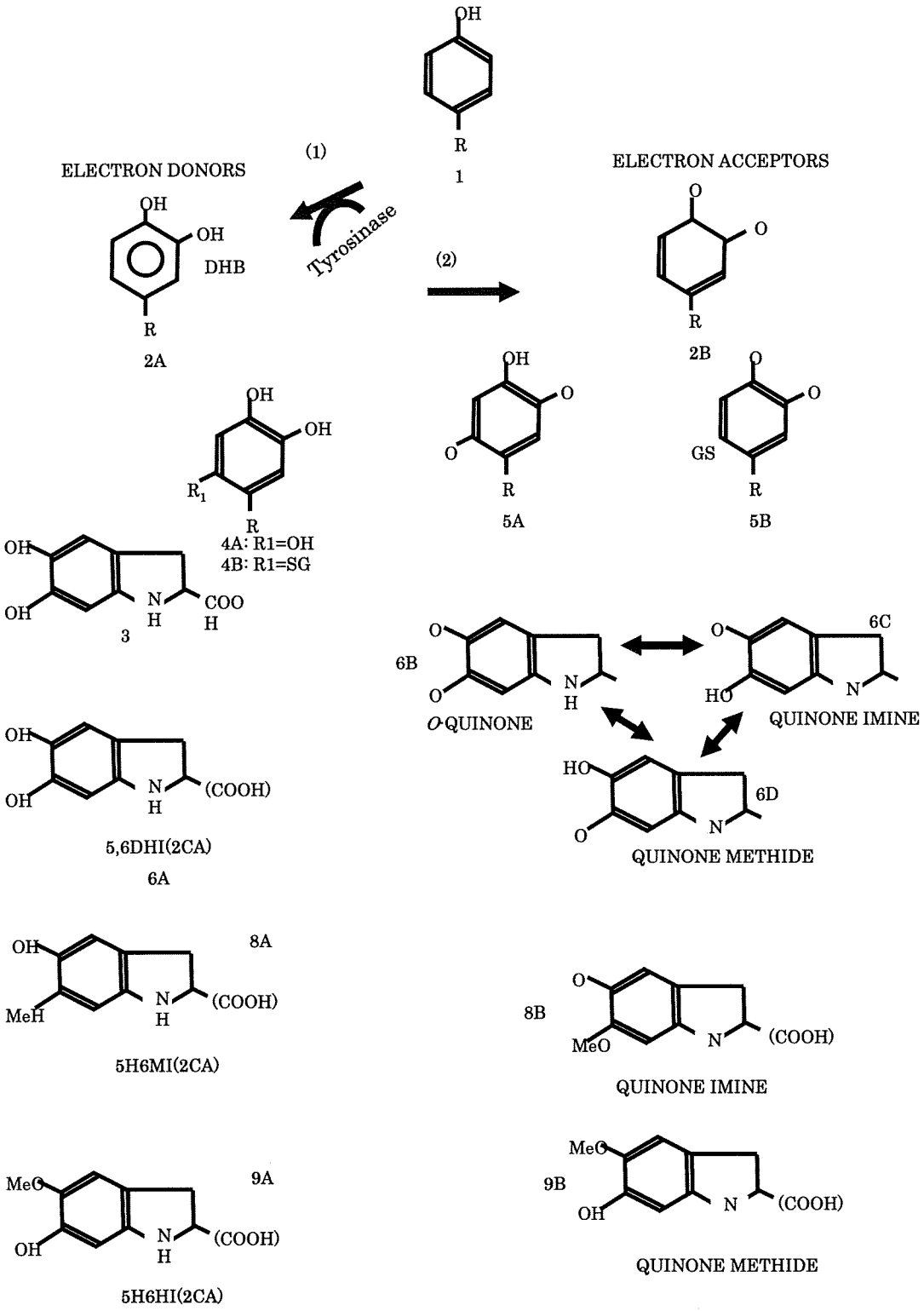
droquinone is an obligatory intermediate. Electron paramagnetic resonance (EPR) studies have shown that phenoxyl radicals can oxidize catechol to 1, 2-benzosemiquinone radical.[29] Phenoxyl radicals oxidize NADH and ascorbate, and they react with superoxide radical and microsomal electron transport system .[28,29,49] Phenoxyl radicals dimerize through the formation of C-C and C-O bonds. These dimmers are better electron donors than the starting phenols.[37,48]

In eumelanin pigmentation, 5,6-Dihydroxyndole-2-carboxylic acid (5,6 DHI 2 CA) and its decarboxylated analog, 5,6 DHI (Scheme 6 A), are produced during melanin pigmentation. By the action of the enzyme *O*-methyl transferase (or dopachrome tautomerase), these dihydroxyindoles may be transformed into mono- and dimethoxylated derivatives, 5 H 6 MI (2 CA), 5 M 6 HI (2 CA), and 5,6 DMI (2 CA), respectively (Scheme 8 A and 8 B). Oxidation of these indoles may lead to free radical of formation. 5,6 DHI, 5,6 DHI 2 CA, and 5,6 dihydroxyindole-1-methyl (5,6 DHI 1 Me) gave rise to semiquinone and semiquinone imine cation radicals when oxidized by radiolytically produced azidyl radical, $N_3\cdot$.[1,31] Free radicals derived from carboxylated


ELECTRON DONORS
(1)
Tyrosinase
ELECTRON ACCEPTORS
(2)
DHB
2A
2B
5A
5B
4A: R1=OH
4B: R1=SG
3
5,6DHI(2CA)
6A
6B
O-QUINONE
6C
QUINONE IMINE
6D
QUINONE METHIDE
8A
5H6MI(2CA)
8B
QUINONE IMINE
9A
5H6HI(2CA)
9B
QUINONE METHIDE

DHI are more stable than radicals from decarboxylated DHI. The increased stability may be the consequence of the presence of the ionized carboxylic group in the molecule. 5,6-Idole quinine, quinine imine, and quinine methide (Scheme 6 B, 6 C, and 6 D, respectively) are found to be the secondary products of 5,6 DHI oxidation. [28] In Scheme 6 B was formed by dismutation of the initially produced semiquinones and then rearranged to 6 C and 6 D. Decay of quinones was followed by the formation of trihydroxyindole derivative THI (Scheme 7). Using selectively methoxylated hydroxyindoles, the intermediate responsible for the formation of THI was identified to be quinine methide (Scheme 6 D). [32]

Thus, based on radiolytic reactions, melanin pigmentation process may be viewed in such a way that the melanin synthesis is initiated by the formation of semiquinone radical from a dihydroxyindole followed by its transformation to quinine indole, its tautomers, and THI product. THI reacts rapidly with 5,6-indolequinone, quinine imine, or quinine methide to give rise to dimmers or oligomer products. In contrast to the mechanism of melanin formation suggested by photochemical reactions, this model of melanin synthesis does not attribute any essential role to phenoxyl radicals. [9,31]

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 quinine and $O_2^{\cdot-}$. Semiquinone radicals can also be produced in the melanogenic pathway non-enzymically, through mechanisms involving: (a) disproportion of quinine 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. In addition, UVR/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 or psoralen plus UVA, PUVA). These processes are partly responsible for the UV light-stimulated cytotoxicity (Fig. 3). [43]

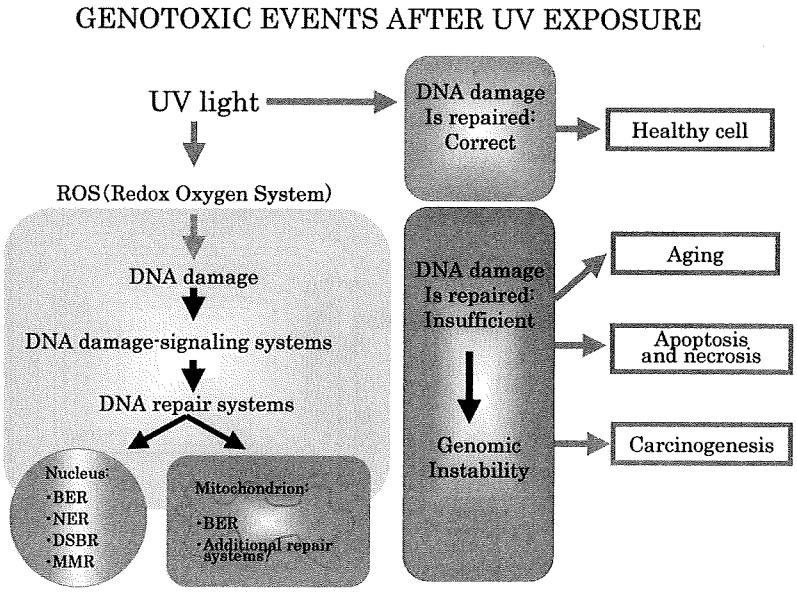


Figure 3: UV-induced genotoxic effects. UVR causes always the DNA damage to some extent. If DNA damage is repaired insufficiently, it will result in either apoptosis or necrosis of cells which will accentuate the aging process. It will also result in genomic instability which may become carcinogenic.

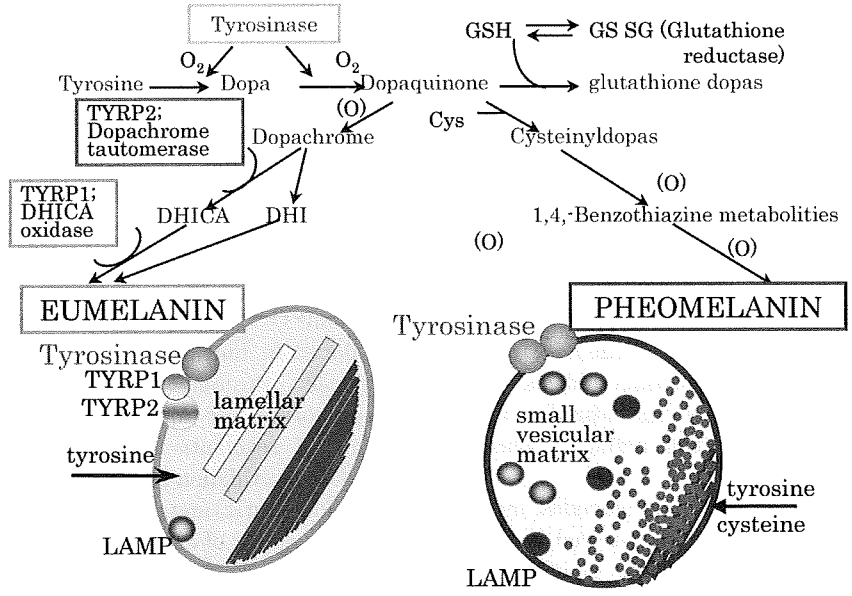


Figure 4: Cascade of eumelanin and pheomelanin biosynthesis. Two forms of melanin pigment are present in mammals. Eumelanin is synthesized by conversion of tyrosine to dopa and dopaquinone in the presence of tyrosinase. Through the interaction of TYRP 1 (DHICA oxidase) and TYRP 2 (dopachrome tautomerase: DCT) or auto-oxidation of dopa quinone, eumelanin will be produced within ellipsoidal granules that contain lamellar matrix inside. In contrast, pheomelanin will be produced after binding of dopaquinone with cysteine to form cysteinyldopa that will be auto-oxidized to form benzothiazine metabolites and result in pheomelanin in oval granules that contain small vesicular matrix.

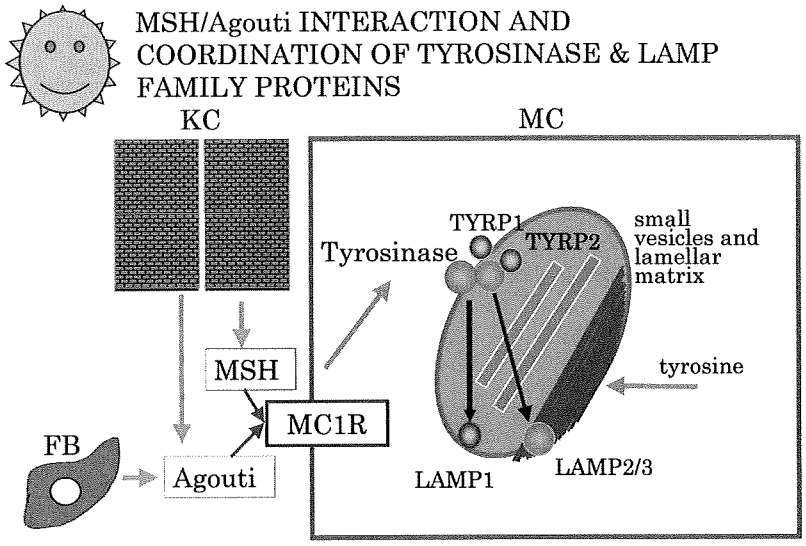


Figure 5: MSH/Agouti signaling protein interaction and coordinated expression of tyrosinase and LAMP family proteins after exposure to UVR.

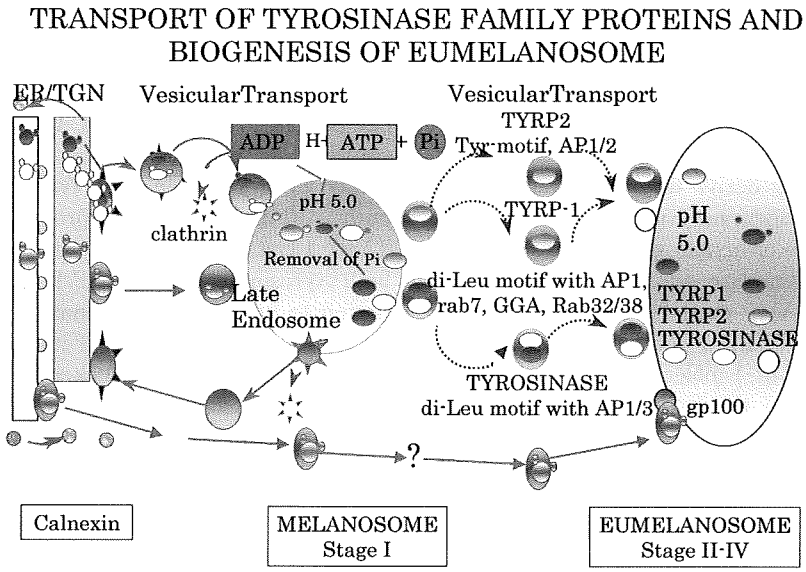


Figure 6: Transport of tyrosinase family proteins and biogenesis of eumelanosomes. Tyrosinase family proteins including TYRP 1 and DCT will transported from Golgi after glycosylation maturation in the presence of molecular chaperone, calnexin, by vesicular transport to late endosomal compartments which are delineated to Stage I melanosomes. Tyrosinase, TYRP 1 and DCT will take the different vesicular transport to Stage II melanosomes in which melanin biosynthesis starts.

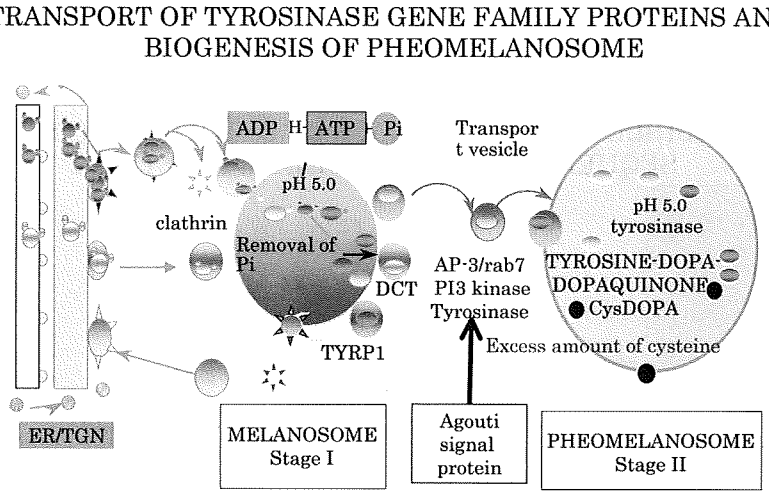


Figure 7: Vesicular transport of tyrosinase and biogenesis of pheomelanosomes.

2. Tyrosinase gene families and melanin biosynthesis

Besides tyrosinase, the major melanogenesis enzyme, converting tyrosine to dopa and subsequently to dopaquinone, two molecules are related to tyrosinase and are referred to as tyrosinase-related proteins (TYRPs in humans and Tyrps in animals) (Fig. 2, 4) ; (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 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 other research groups have identified lysosome-associated membrane protein (LAMPs) which are associated with the membrane of the two granules.[34,39] 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 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 (Fig. 4, 5).[20,21,40]

Both tyrosinase and TYRP 1 are transported from the trans-Golgi network (TGN) to melanosomes via endosomal compartments (Fig. 6, 7). Adaptor proteins (APs) generate transport vesicles, sorting proteins and assembling clathrin to the local membrane in endosomes.[42] AP 3, one of the four known mammalian APs, has been reported to bind to a dileucine motif of tyrosinase.[5,14] A mutation of β 3 A subunit in AP 3 results in Hermansky-Pudlak syndrome (HPS)-2 in humans and pearl in mice.[8] Accumulating evidence suggests that both tyrosinase and TYRP 1 interact with APs and travel from endosomes to melanosomes, though the exact transport pathway is still not clear. It has recently been reported that tyrosinase can use both AP 1 and AP 3 for its proper sorting while TYRP 1 can use only AP 1.[53] It is also clear that both tyrosinase and TYRP 1 are not transported to lysosomes. Tyrosinase may, however, be dedraded in lysosomes if its transport from TGN to melanosomes is blocked.[10]

3. Eumelanin biosynthesis and biological role of tyrosinase-related proteins

Mutations that effect different stages of melanocyte development have been best characterized in experimental mice. Both albino mutation of mouse tyrosinase and brown mutation of tyrosinase-related protein 1 (Tyrp-1) alter cysteine in the EGF motif to serine in albino mutation and different cysteine to tyrosine in brown mutation. Proof of the mutation status of the alteration has, in both cases, 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

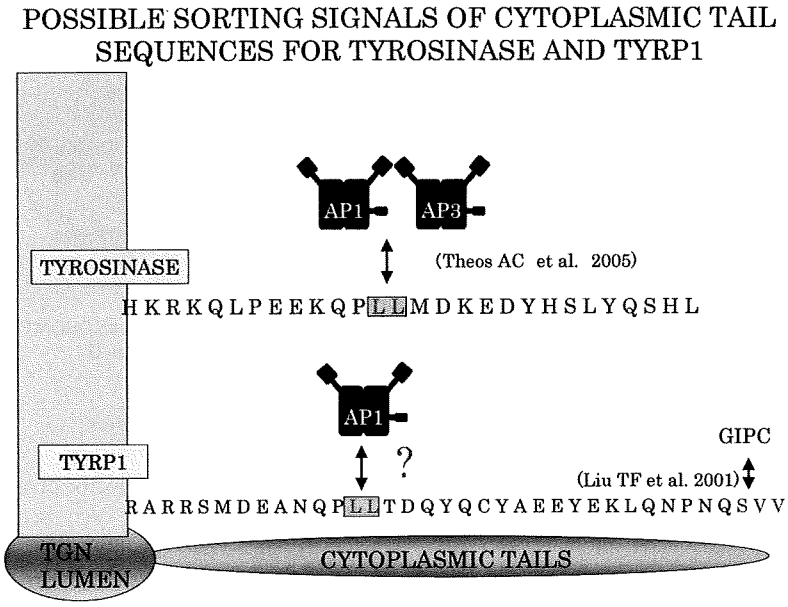


Figure 8: Possible sorting signals of cytoplasmic tail sequences for tyrosinase and TYRP 1. Cytoplasmic tail motifs of tyrosinase and TYRP 1 are referred from the reports of Theos AC et al. and Liu TF et al. respectively.★21,36)

that both mutations have complete loss of function of the gene product. On the other hand, Tyrp-2 containing the slaty mutation, which has arginine to glutamine change at the first copper -binding sites may still have considerable DCT activity (Fig 8).

The function of TYRP/ tyrp-1 is still not fully understood, however, it is believed to be involved in eumelanin synthesis since the brown mutation only affects the eumelanic animals. It has shown that demonstrated gene changes in Tyrp-1 mRNA expression and in Tyrp-1 gene structure in certain mouse brown b-locus mutants. 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 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 rearrangement, 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.[20,23]

4. Pheomelanin biosynthesis and its elevation in melanoma and dysplastic nevi

The diverse patterns of mammalian coat color are determined by the quantity and distribution of just two types of organic pigment: eumelanin (black to brown) and pheomelanin (yellow to red). Both are produced by melano-

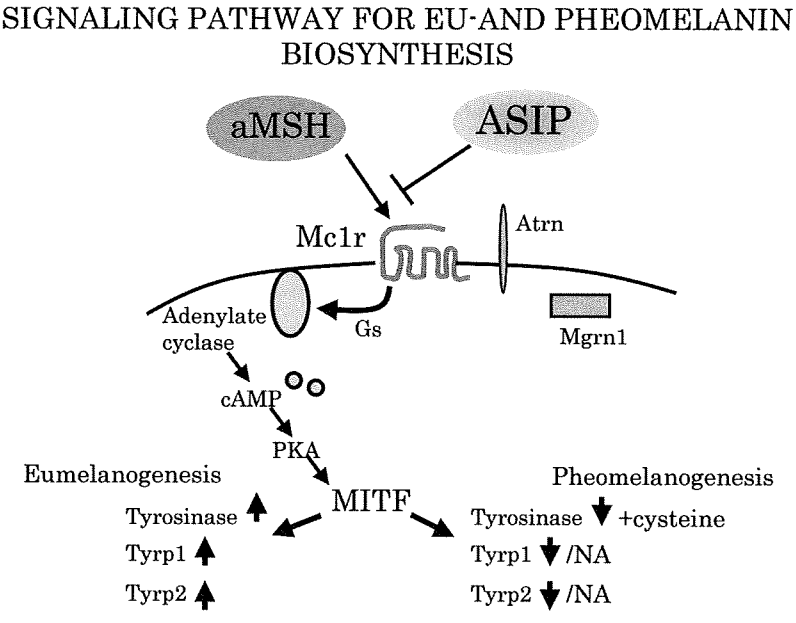


Figure 9: Biological role of agouti signaling protein (ASIP). Agouti signaling protein is the key protein molecule which is involved in pheomelanin biosynthesis and which antagonizes to largely unknown MSH binding with Mc 1 r (melanocortin 1 receptor) on the melanocyte cell membrane. Attractin (Atrn) binds N terminal of Agouti/ASIP and supports this binding to Mc 1 r. Biological role of mahogunin (Mgrn) is still unknown.

cytes in the skin and hair.[20,21] Two major loci are central to pigment-type switching in mouse. One is the agouti locus encoding agouti signal protein (ASIP), with mutants including non-agouti (*a*), giving a eumelanic black mouse in the absence of other mutations) and dominant yellow (*Ay*) ; the other is the melanocortin-1 receptor (*Mc 1 r*) locus, formerly extension (*e*), also with both eumelanic and pheomelanic mutants (e.g. recessive yellow, $Mc1r^{e}$) (Fig. 9). MC 1 R is a cell-surface G-protein-coupled receptor for which the best-known agonist is the soluble peptide αMSH, cleaved from the precursor pro-opiomelanocortin (POMC) in the pituitary and skin. Binding of αMSH to MC 1 R is known to activate adenylate cyclase and cAMP synthesis, promoting eumelanin synthesis through both post-translational and transcriptional pathways via microphthalmia-related transcription factor (MITF).[3] 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 SILV/PMEL. Synthesis of both eumelanin and pheomelanin start from tyrosine oxidation catalyzed by tyrosinase. The resulting dopaquinone can be a substrate for eumelanin synthesis, promoted by TYRP 1 and DCT, or pheomelanin in the presence of high cysteine concentrations and/or low tyrosinase activity.

ASIP is a soluble protein of 131 amino acids, apparently secreted by dermal papilla cells in hair bulbs. It competitively antagonizes αMSH at the MC 1 R and inhibits the eumelanogenic signal, down regulating melanogenic enzymes and leading to pheomelanin synthesis (Fig. 10). For better understanding of ASIP signaling, we have sought to develop culture conditions under which ASIP contributes to overt pheomelanin synthesis by the melan-a immortal murine melanocyte line.[11] It was demonstrated that ASIP signaling can be reduced melanocyte growth and induce morphological dedifferentiation as well as affecting pigmentation (Fig. 10). These biological effects are mimicked in genetically yellow melanocytes, incomplete in Atrn- and *Mgrn1*-null melanocytes, appear independent of cAMP down-regulation, and require the amino terminus of ASIP.

As discussed in our previous report [18], significantly high content of pheomelanin was found in melanoma and dysplastic melanocytic nevi (DMN) lesions by analysis using high-pressure liquid chromatography (HPLC)[2,4,15-17,26,45], in-

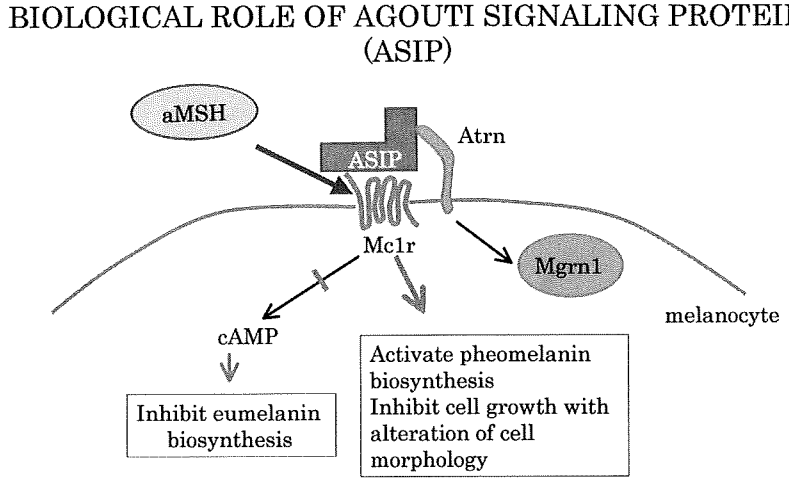


Figure 10: Biological role of agouti signaling protein (ASIP). Activation of ASIP will result in not only eumelanin production inhibition and pheomelanin production activation but also the differentiation and proliferation of melanocyte. It is likely that there is an alternating signal transduction cascade beside cAMP cascade in this process.
This un-clarified, new cascade passes through Mc 1 r but is independent to cAMP cascade and does not include the pathway of Attractin (Atrn)/ mahogunin 1 (Mgrn 1).

dicating that these abnormal melanosomes may be pheomelanic.[11,52] Our previous electron microscopic studies showed that melanocytes of dysplastic melanocytic nevi DMN were rich in abnormal melanosomes which exhibited features consistent with pheomelanosomes. It would be important to examine the type of melanogenesis in abnormal pigmentation in the aged skin such as senile freckle and solar lentigo. [11]

3. UV-photoproducts of pheomelanin and its precursors, and enhancement of skin aging process

Catalase structure and activity was seriously affected by photo-oxidation of its own substrates, hydrogen peroxide, owing to cleavage of its porphyrin active site.[2,51] 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 process.[47,54] UVA-irradiated pheomelanin altered the structure of catalase and decreased its activity in human skin.[35] Electron-spin resonance spectroscopy experiments by Sealy et al. on black and red melanin, suggested that red melanin (pheomelanin) contained a specific kind of free radical (s) not present in black eumelanin.[46]Chedekel et al. found that pheomelanin was photo-destroyed, in the presence of oxygen, by UVR.[5] Haryanvi et al., using the reversion test of Ames, demonstrated that pheomelanin became mutagenic after exposure to UVR.[11] Koch, Chedekel and Meresca et al. described photo-initiated DNA damage by melanogenic intermediates of 5-S-cysteinyldopa origin.[30,35] The binding of these molecules to DNA was activated by 300 nm UVR and resulted in single-strand breaks. However, while biosynthesis process and biological role of eumelanin after exposure to UVR are well characterized, the nature and photo-biological role of pheomelanin may still be largely unknown.

Summary and Perspective

Melanin pigmentation of the skin has been generally regarded to possess a protective role against exposure to UVR. Melanin pigment, specifically eumelanin, may therefore provide a protective role against development of sunburn, solar degeneration and skin aging as well as cancer.[24,25] Evidence accumulated recently indicates that the photoprotective role of the melanin pigment may be restricted to eumelanin as well as to the visible and UVA ranges and that in the UVB and UVC ranges both eumelanin and pheomelanin components may behave as photosensitizers. UV photolysis studies using catechols, catecholmines, and hydroxylated indole derivative (i.e., the eumelanin precursors) have demonstrated the formation of hydrated electrons, hydrogen atoms, semiquinone/ semiquinone imine radicals, and indoloxyl radicals. The photolysis of pheomelanin precursors leads to the formation of hydrated electrons, hydrogen atoms, and to alanyl and aryl thiyl radicals. Thus, while eumelanin may act as a photoprotector, its precursors may be still phototoxic.

The photoprotection by melanin pigment can be accomplished by scavenging the reactive free radicals, by quenching excited states, and by decreasing oxygen concentration in exposed tissues. The phototoxicity is related to the generation of reactive free radicals by low molecular eumelanic and pheomelanic intermediates. It may, however, be possible to modulate the photosensitivity of the pigmentary system by affecting the redox status of endogenous melanin pigments, or by switching melanin pigmentation from eumelanogensis to pheomelanogenesis, or *vice versa*, or by using sensitizers activated by red light, which is poorly absorbed by melanin.

Further investigation of the biological role in eu- and pheomelanin biosynthesis after UVR is required to provide a new insight for the better understanding the skin aging process.

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

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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

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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.

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

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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。

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

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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

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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.

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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.