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INVITED REVIEW

EXPLOITATION OF MELANIN AND MELANOSOME BIOGENESIS FOR BETTER MANAGEMENT OF MALIGNANT MELANOMA

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ABSTRACT

Management of malignant melanoma is a difficult challenge for physicians and basic scientists because cutaneous melanoma grows in the highly vascular tissue of epidermo-dermal interface and occurs at any age of generation. It can easily spread to distant organs and practically no treatment modality is available except for early diagnosis and surgical excision. In this report we review our current research efforts in establishing novel diagnostic and therapeutic approaches for malignant melanoma by exploiting our knowledge of basic biology in melanin and melanosome biogenesis. This is based upon the fact that melanin and melanosome biogenesis is biological property uniquely expressed in melanocytes, and highly accelerated and altered in their neoplastic counterpart, malignant melanoma. It is immediately apparent if one looks at the past literatures that rapid and significant progress of cancer management has been made if the biological property unique to cancer cells is exploited.

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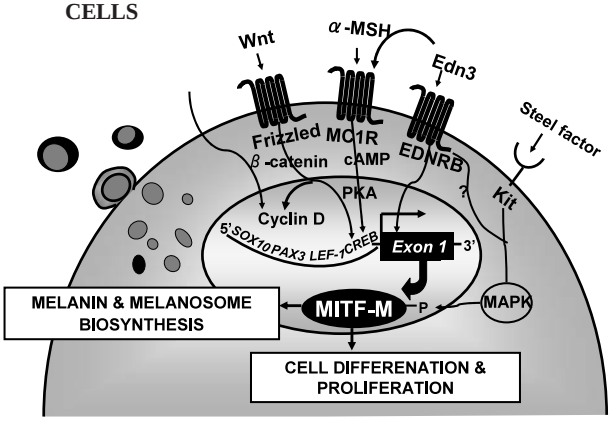
Key words: Melanoma, Skin cancer, Melanin, Melanosome, Chemotherapy, Immunotherapy, Epidemiology, Nano-medicine

1 INTRODUCTION

This review is aimed to introduce our current research effort in exploiting our knowledge of melanin and melanosome biogenesis for better developing diagnostic and therapeutic approaches to malignant melanoma. In this review we cover four major topics that include (1) melanogenesis cascade and melanoma biology, (2) transcription factors and melanoma epidemiology, (3) tyrosinase family proteins and melanoma immunotherapy, and (4) melanin cytotoxicity and melanoma nano-medicine.

Malignant melanoma cells take two basic patterns of proliferation, i.e., horizontal radial growth along the epidermal dermal interface and perpendicular vertical growth in which melanoma cells migrate downward from the epidermis to the dermis. Three forms of melanomas, i.e., lentigo maligna, superficial spreading and acral lentiginous types, take this radial growth. The clinical behavior and histopathological features of these three melanoma subtypes are unique and distinct to each other. These unique clinical behavior and histopathological fea-

Fig.1 TRANSCRIPTION FACTORS AND RELATED PROTEINS OF MELANOCYTES AND MELANOMA CELLS



MITF:Microphthalmia associated transcription factor

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tures are regulated by many factors, such as Wnt signals, melanocyte stimulating hormone (MSH), endothelin, steel factor and microphthalmia-associated transcription factor (MITF). These factors and hormone are connected in their downward stream of signaling cascade to MITF, which will then activate the gene expression of tyrosinase, tyrosinase-related protein (TYRP) 1 and 2 as well as melanosomal structural protein, gp100[1](Fig. 1).

2 RESULTS AND DISCUSSION

2.1 Melanogenesis Cascade and Melanoma Biology

2.1.1 Melanin biosynthesis

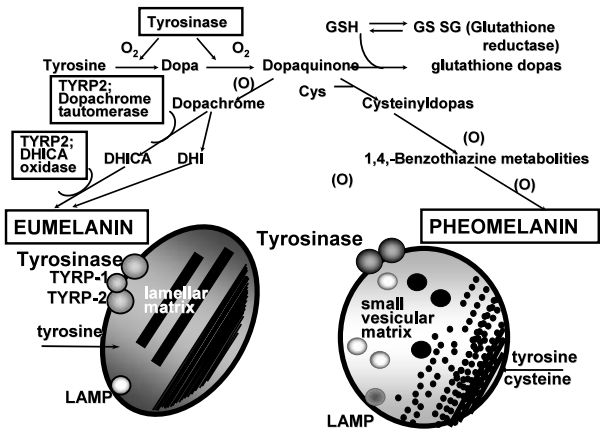
The biosynthesis of melanin pigments occurs in secretory granules, melanosomes. In the presence of tyrosinase, tyrosine is converted to dopa and dopaquinone. Dopaquinone is further oxidized to form dopachrome, which is then oxidized to form eumelanin either through auto-oxidation or in the presence of two tyrosinase-related proteins (TYRP1 and 2) that have dopachrome tautomerase and DHICA oxidase activities respectively. In contrast, when dopaquinone encounters cystein, cysteinlydopa is formed, and then through auto-oxidation, cysteinyldopa is converted to benzothizine metabolites to form pheomelanin. Tyrosinase is therefore the key enzyme being involved in both eu-and pheomelanin biosynthesis[2](Fig. 2).

2.1.2 Identification of a molecular chaperon for maturation and transport of tyrosinase family protein

In our study of immunoscreening of human melanoma cDNA expression library using an anti-human melanosome antibody, we found a number of melanosome/or melanogenesis-associated proteins in addition to tyrosinase. One of them was calnexin. Calnexin is a type I membrane protein, functions as a molecular chaperon which associates specifically with incompletely folded or unassembled glycoproteins and facilitates their acquisition of correct three-dimensional protein structure. Tyrosinase, at the beginning of the synthesis in the ER, consists of triglucosylated core oligosaccharide that will become, by the sequential action of glucosidase I and II monoglucosylated asparagine-like oligosaccharide, for which calnexin shows affinity. If the molecular chaperon function of calnexin is critical for proper folding of tyrosinase, inhibition of glucosidases in the ER should abolish tyrosinase activity[3-4]. We have studied the effect of such an inhibitor, castanospermine (CST) on tyrosinase expression. In the absence of CST treatment, the immunoprecipitation of tyrosinase and calnexin cDNA infected cells showed both tyrosinase and calnexin bands by anti-calnexin antibody. However, in the presence of CST treatment, those cells infected with both tyosinase and calnexin cDNAs showed only calnexin band, indicating the complete block of tyrosinase binding with calnexin. Furthermore, tyrosinase band after treatment with CST moved more slowly than that without CST treatment because of immature folding of tyrosinase. Furthermore, CST treatment completely abolished the tyrosinase activity and melanin synthesis[5].

The functional role of calnexin for tyrosinase maturation and activity as well as melanin synthesis was further characterized by employing a heterologous expression system of wild-type and cyt (cytoplasmic tail)-deleted calnexins and wild-type tyrosinase cDNAs in COS7 cells. Three groups of cells were infected with control pcDNA, wild type or cyt-deleted calnexin. They revealed comparable immunoprecipitation band of tyrosinase infection. COS7 cells have some endogenous expression of calnexin, therefore the cells infected with control pcDNA or cyt-deleted calnexin with wild-type tyrosinase cDNAs showed some tyrosinase activity and melanin synthesis that occurred in lysosomal or endosomal compartments. In contrast the cells incorporated with wild type calnexin and tyrosinase cDNAs showed tyrosinase activity and new melanin synthesis which continued to rise up to 96 hr after tyrosinase infection. On the other hand, cells infected with cyt-deleted calnexin cDNA showed a minimal increase of tyrosinase activity and melanin synthesis, at least a half expression of tyrosinase activity and melanin synthesis by cells infected with control pcDNA[5].

Fig.2 BIOSYNTHETIC PATHWAY OF EUMELANIN AND PHEOMELANIN



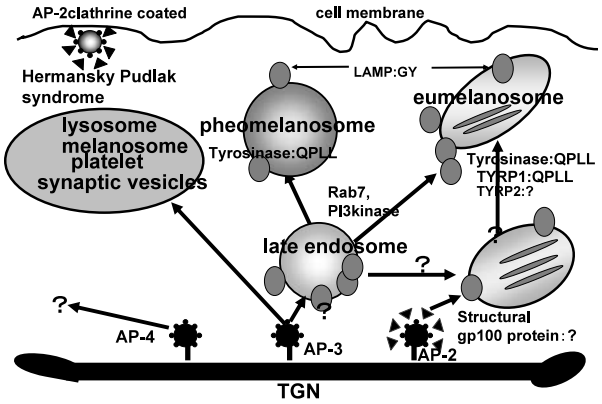
2.1.3 Identification of target signals and transporters of tyrosinase family proteins

Our next step for the export or transport process of tyrosinase family proteins from Golgi to melanosomes is the identification of their target signals. For this purpose, a number of human TYRP1 mutants in the cytoplasmic domains were synthesized. Mutant sequences were made based upon the known target signals of endosomal/lysosomal protein because melanosomes and lysosomes share many common structural and biological properties. Two distinct motifs of the cytoplasmic tail, i.e., tyrosine-rich dileucine and YQRL were found to be the target signals from TGN or cell surface to endosomal or lysosomal granules. Mutant cDNAs of either deletion or substitution at the dileucine (LL) or tyrosine (Y) sequences of c-terminal were constructed and transfected to human amelanotic melanoma cells, which lacked both gene and protein expression of tyrosinase family, but formed non-melanized melanosomes. The TYRP-1 infected cells were double-labeled with the antibody against either cation-independent mannose-6-phosphate receptor (M6PR) or lysosome-associated membrane protein (Lamp-1), which is a late endosomal or lysosomal marker. Newly synthesized TYRP-1 was found to be co-localized with M6PR-positive granules that were present in the centre of the perikaryon. Importantly the previously defined stage I melanosome was found to be delineated to this late endosomal compartment. Late endosome is the convergence site of either endocytosed material from early endosome or newly synthesized lysosomal or secretory protein exiting TGN through early endosome[6-7].

When tyrosine sequence of TYRP-1 was either deleted or substituted with serine, there was no significant alteration in the distribution of newly synthesized M6PR-postive TYRP-1 granules, indicating that tyrosine sequence does not play any role in providing a target signal to TYRP-1 for intracellular trafficking. In contrast, when dileucine sequence was either deleted completely or substituted with serine, or only one leucine was substituted, there was no granular distribution of newly synthesized TYRP-1. Our finding indicated the importance of dileucine sequence at the c-terminal for the target signal of TYRP-1 to melanosomes. It is also known that tyrosinase requires dileucine motif as a targeting signal from the study of drosophila eye pigmentation[8].

Other transporters that tyrosinase family proteins require for vesicular transport from TGN to melanosomes are adaptor proteins (APs). There are four APs identified so far. AP-3 was found to be bound to the tyrosinase cytoplasmic tail, which contained an EESSLL dileucine sequence. AP-3 does not bind to TYRP1 but rather interacts with AP-1 or AP-2. There must be also other adaptor proteins that interact with structural matrix protein such as gp100. Further studies are needed to characterize the interaction between sorting signals of melanosomal proteins and APs (Fig. 3).

Fig.3 VESICULAR TRANSPORT OF TYROSINASE FAMILY AND STRUCTURAL PROTEINS IN MELANOSOME BIOGENESIS



2.1.4 Small GTP binding proteins and PI3kinase for vesicular transport

Other proteins necessary for trafficking of tyrosinase family protein from TGN to melanosome include small GTP binding proteins and PI3 kinase that are identified from our fractionation study of melanosomal proteins[9]. This review covers small GTP-binding proteins. In order to identify novel proteins associated with melanosome biogenesis, we have purified the native melanosomes and fractionated their melanosomal proteins on 2D SDS-PAGE. Approximately 60 protein spots were identified from melanosomal proteins of B-16 melanoma cells. One of them, #12 spot was always seen, and its amino acid sequence was identified and converted to DNA sequence, which was then amplified by RT-PCR mediated molecular cloning. This nucleotide sequence revealed a high homology with small GTP-binding protein. A GTP-overlay assay was carried on 2D-SDS PAGE of melanosomal proteins and at least 5 to 6 spots were identified within the range of MW 19-27 kD. Based upon the search on GTP-binding proteins with similar MW and pI, Rab 3, Rab 7 and Rab 8 were identified, and finally Rab7 was found to be the protein involved in the transport of TYRP1 from Golgi to melanosomes. The co-localization of Rab 7 with TYRP-1 on melanosomal com-

partment was verified by superimposing the two images of double-immunostaining reactions for Rab7 and TYRP1 using confocal microscopy[10].

The involvement of endosomal transport of TYRP1 to melanosomes was further examined by anti-sense and reverse anti-sense Rab 7 oligonucleotide experiment. In this experiment, we first confirmed the efficiency and specificity of Rab 7 expression in various melanoma and non-melanoma cells by immunoprecipitation. We then observed the cells treated with anti-sense and reverse anti-sense to Rab 7. Rab 7 anti-sense group showed a significant, at least 30 - 50 %, decrease of Rab 7 expression compared to that in the cells with reverse anti-sense Rab7. Finally we found strong perinuclear staining of TYRP-1 and faint or no staining of TYRP-1 in the periphery of perikaryon in cells with reverse Rab7 anti-sense treatment, indicating the impairment of TYRP-1 transport from the perinuclear rim to melanosomes.

The involvement of Rab 7 for intracellular transport of tyrosinase family protein was also confirmed by the comparison of the expression of TYRP-1 in the presence or absence of dominant/negative Rab 7 mutant (Rab 7 N125I). Newly synthesized TYRP-1 was found to be blocked for being transported to lgp 85 (a lysosome marker) positive compartments, i.e., from 49.4% to 17.9% co-localization. The same result was obtained when the cells were stained with anti-syntaxin 8, a marker for the late endosome compartment. The blockade of newly synthesized TYRP-1 in dominant/negative Rab 7 mutant appeared to be segregated in early endosomes as could bee seen in the staining of EEA1, an early endosome marker. These sense/anti-sense Rab 7 oligonucleotide and dominant/negative Rab 7 experiments show clearly the mandatory requirement of Rab7 for vesicular transport of TYRP1 in melanogenesis[11].

2.2 Transcription Factors and Melanoma Epidemiology

2.2.1 Melanocortin receptors and melanoma development

Melanocortin receptor (MCR) family comprises five members. They are distributed to various kinds of cells. MC1R has been found on epidermal melanocytes and melanoma cells as well as on various kinds of cells such as keratinocytes, microvascular endothelial cells, human dermal fibroblasts, mast cells, macrophages, follicular epithelia, sebaceous glands and secretory and ductal epithelia of sweat glands. Aside from MC1R, MC2R is expressed almost exclusively on adrenal gland cells, MC3R on central nervous cells or macrophages, MC4R on central nervous cells and associated with regulation of appetite, and MC5R on many kinds of exocrine gland including foreskin, lacrimal, sebaceous and eccrine glands. MC1R responds mainly to alpha-MSH.

MC1R locates on the cell surface of the melanocytes and melanoma cells, and regulates eu-and pheomelanogenesis through the interaction with either MSH (melanocyte stimulating hormone) or agouti signal protein. Its major role in the normal human skin is the interaction with MSH which is generated by epidermal keratinocytes after exposure to the sun light. After binding with MC1R, alpha MSH stimulates the intracellular cascade of cAMP and PKA which then phosphorylates a transcription factor, CREB (cyclic AMP responsive element binding protein) and then activates MITF that subsequently binds the upstream regions of genes expressing tyrosinase and tyrosinase-related proteins, i.e., TYRP1 and 2 (dopachrome tautomerase; DCT) to stimulate melanin synthesis[12]. MC1R contributes to individual skin or hair color. The diversity of coat color of mammals represented by mouse hair or human skin and hair refers to the MC1R function which makes a difference of quality and quantity of melanin production. Eumelanin is a black-brown pigment while pheomelanin is a yellow-red one and the proportion of these two kinds of melanin determines the human hair and animal coat color[2].

2.2.2 MC1R polymorphism in Japanese melanoma

In mice, the synthesis of pheomelanin is under the regulation of *agouti* gene coding for the agouti signaling protein, the physiological antagonist of alpha-MSH. Although neither agouti gene nor agouti signaling protein has been found yet in human, the diversity of melanin production has been confirmed to depend partly on MC1R. So far more than 65 variants of MC1R gene has been reported and some of them had a strong association with skin and hair color. Of the many MC1R gene variants, Arg151Cys, Arg160Trp and Asp294His have been described as reliable candidates for the responsibility of red hair and fair skin in Caucasian. As to other variants such as Val60Leu, Asp84Glu, Arg142His, the correlation with hair or skin color is controversial or rather weak, if any. Other frequent variants, Val92Met, Arg163Gln and Thr314Thr may have prevalence in specific regions or populations, but no significant association of these variants with light hair and skin colors and/or susceptibility to melanoma development has been reported. Since MC1R plays a key role for pigmentation, it would be possible that MC1R is involved in pigmentary phenotypes includ-

ing freckle, photosensitivity, development of nevus and carcinogenesis. Some active MC1R variants were reported to associate with skin types, freckle, melanoma and basal cell carcinoma or squamous cell carcinoma, whereas no variant was presented to associate with the number of moles.

2.2.3 Val92Met MC1R variant as a candidate for Japanese non-acral melanoma

In our recent study, we have found that (1) an MC1R variant, Val92Met, which had been frequently reported previously as normal, revealed a statistical significance for melanoma cases compared with controls ($p = 0.048$), and (2) no variants described previously as susceptible to melanoma in Caucasian were detected in Japanese melanoma. Arg163Gln was observed in 97.9% of total subjects so that this variant must be a wild type allele in Japanese. As the similar results on frequency of Arg163Gln were reported previously in Korean or in American Indian, this variant may form a peculiar distribution of races according to genetic evolution. In our study, Val92Met substitution did not show higher frequency in melanoma cases than in previous reports as an Asian population but did show lower frequency in controls, consequently deriving an association to melanoma susceptibility in Japanese. Considering no other characteristic features including clinical subtypes of melanoma could link with Val92Met except that it harbored a preference for non-ALM melanoma, the background or conclusions would not be drawn immediately yet. Val92Met has been found in a certain level of population around the world and described not to affect the pigmentation-related phenotypes except for a few studies. A strong association of Val92Met with skin type was reported. Val92Met was found to correlate to melanoma patients limited to skin type III or IV, and a higher ratio of Val92Met was seen in vitiligo patients than in controls. Furthermore, the argument about Val92Met on the activation of MC1R *in vitro* is in controversy. Several reports concluded that Val92Met did not exhibit loss-of-function of MC1R whereas one study presented it for decreased synthesis of eumelanin resulting in red hair and burning tendency of type I and II. Our result, however, may be supported by the study of Scott *et al* (2002)[13] showing that cAMP level was significantly increased and cell number was also increased with dose-dependent manner of alpha-MSH using the cultured melanocytes with Val92Met compared to the one expressing wild-type. This evidence may allow a possible hypothesis that Val92Met provides the MC1R function to proliferate melanoma cells *in vivo* in Japanese[14].

2.2.4 Cyclin D1 and melanoma progression

In order to identify the new prognostic markers for malignant melanoma, we have carried out the DNA microarray comparison of primary and metastatic melanoma lesions in the same patients. We found that melanoma inhibitory activity (MIA), S-100 Ca^{2+} binding protein α and β, Wnt inducible signaling pathway protein 2, transcription factor AP-2α, and cyclin D1 are amplified higher in primary melanoma than in metastatic one. In contrast, small inducible cytokine subfamily A, stromal cell derived factor 1, transgelin and frizzled-related protein are amplified higher in metastatic melanoma than in primary one. Among these expressions, Wnt inducible signaling pathway protein and cyclin D1 maybe significant, as it has been recently shown that transcription factors and related proteins are intimately related to melanoma development and proliferation. Specifically Wnt signaling protein directly affects cell mobility and invasion of metastatic melanoma. Cyclin D1 is one of the target genes of Wnt signaling pathway. Wnt signal activates beta-catenin after binding with the frizzled membrane receptor. Beta-catenin then forms a complex with APC and Tcf/Lef, and then is translocated into nuclei and activates transcription of target genes such as cyclin D1.

Cyclin D1 promotes G1/S transition of cell cycle by the phosphorylation of Rb protein. Nuclear accumulation of cyclin D1 and Rb protein accelerates the development and histogenesis as well as malignant alteration of melanoma. We have carried out the immunohistochemistry of cyclin D1 and phosphorylated-Rb protein on Japanese melanomas, more than 50% of which are acral lentiginous type. It was found the markers of cyclin D1 and phosphorylated Rb protein are localized in the same melanoma lesions on immunohistochemistry. Statistical analysis using Kaplan-Meier method indicated the parallel relationship in gene expressions between cyclin D1 and β-catenin, and a high nuclear expression of cyclin D1 and phosphorylated Rb proteins to be a poorer prognostic marker for overall survival (Log rank test $p<0.1$). Furthermore this trend was more significant in male patients than females[15].

2.3 Melanosomal Glycoproteins and Melanoma Immunotherapy

2.3.1 Dynamics of tyrosinase maturation, segregation and transport

In order to further characterize the dynamics of tyrosinase maturation in the ER and processing to Gogi and subsequent export from Golgi to melanosomes, we have developed a system in which maturation and processing of tyrosinase is temporarily arrested in TGN through shifting the culture temperature from 37 to 40 ℃ by utilizing the unstable folding properties of tyrosinase with heat exposure and the interaction of this unstable tyrosinase with calnexin[15]. The inactivation of tyrosinase activity incubated at 40 ℃ is recovered soon after 37 ℃ re-incubation of culture cells and reaches plateau within 1 hr. Furthermore, this arrest of tyrosinase export at 40 ℃ is recovered within 2 hrs at 37 ℃ incubation, and tyrosinase is properly exported from Golgi to endosome/lysosomes system upon temperature shift. We, therefore, succeeded to establish a system by which the dynamics of newly synthesized tyrosinase trafficking from the ER to Golgi and then Golgi to endosomal or lysosomal granules can be visualized. This system can be used for characterizing dynamics of tyrosinase trafficking. For example, tyrosinase labeled with green fluorescence of YFP was introduced into COS7 cells by fusing YFP to the carboxyl terminus of tyrosinase together with identification of Golgi location by fusion of red-colored CFP to the N-terminal 81 amino acids of galactosyl transferase.

In this system, we found that newly synthesized tyrosinase-YFP is accumulated in Golgi labeled with CFP at 40 ℃ and then placed at 37 ℃. We can clearly see the sequence of events for accumulation of tyrosinase at TGN and its subsequent export from the tip of of tubular structures of Golgi to late endosomal vacuoles at 4'45" to 5'30". Within the next 15 sec, a second projection was observed from Golgi. This tubulovesicular structures lacked Golgi-CFP at 5'45" but revealed Golgi-CFP 15 seconds later at 6'00", indicating the segregated tyrosinase to be exported to late endosomal vacuoles[16].

2.3.2 Misfolded melanosomal glycoproteins as melanoma antigens

In our pulse chase experiment an interaction of newly synthesized tyrosinase with calnexin in the ER was found to be transient. The association of tyrosinase and calnexin was highest immediately after the pulse and nascent tyrosinase was gradually dissociated upon chase. In addition, the proper folding of tyrosinase was largely dependent on its direct interaction with calnexin for the determined duration in the ER. Then what will happen to the sorting of mis-folded tyrosinase as in the case of malignant melanoma. Mis-folding of melanosomal glycoproteins can easily occur in malignant melanoma due to hostile microenvironment. All mis-folded tyrosinase and other melanosomal glycoproteins bind to calnexin in the ER. They will be then eliminated into the cytoplasma where their N-linked glycans will be removed through the interaction with glycanase and degraded by proteasomes. They will be further transported back to ER by TAP transporter and bind with MHC class I heavy chain in ER. This complex of degraded tyrosinase and MHC class I heavy chain is moved through vesicular transport from TGN to the cell surface where antigen presentation to CD8 positive lymphocytes occurs. Degraded cytoplasmic tyrosinase may also be directly accumulated in late endosomal compartment and transported through MHC class II pathway to the cell membrane where antigen presentation to CD4 positive cells may occur. The peptide molecules of tyrosinase and other melanosomal glycoproteins are now widely used for immunotherapy of malignant melanoma.

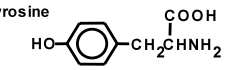
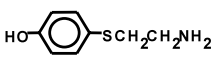
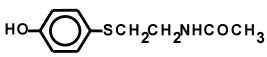
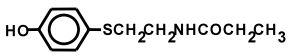
2.4 Melanin Cytotoxicity and Melanoma Nano-medicine

2.4.1 Exploitation of melanogenesis for development of novel chemotherapeutic approach for melanoma

Our goal in this project is to develop melanocytotoxic agents for melanoma chemotherapy and hyperpigmented skin lesions. Our approach is based upon the exploitation of the potentially toxic melanin biosynthesis pathway unique to melanocytes, using sulphur containing tyrosine analogues as pharmacological agents. It is well known that melanin biosynthesis per se, if overproduced, is toxic to melanocytes and may kill them. This cytotoxicity primarily derives from tyrosinase mediated formation of dopa, dopaquinone and other quinone intermediates, which form cytotoxic free radicals. Under normal conditions the melanocyte has the machinery such as TYRPs to withstand this oxidative stress within melanosomal compartments[17]. However, the anti-oxidant defence may break down under conditions such as highly increased melanogenesis substrate analogues entering the pathway. This may result in cytotoxic action of the reactive species leading to melanocyte destruction[17-20].

Based upon this rationale, we have synthesized first

Fig.4 SULPHUR HOMOLOGUES OF PHENOLIC AMINES AS TYROSINASE-TARGETED MELANOCYTOTOXIC AGENTS

	Km (μM)	Vmax (μmole/min/mg)
Tyrosine 	0.3	1.80
4-S-CAP 	117.0	7.97
N-AcetyI-4-S-CAP (NAcCAP) 	375.0	9.28
N-PropionyI-4-S-CAP (NPrCAP) 	340.9	5.43

sulphur homolog of tyrosine, cysteinylphenol and then its amine derivative, cysteaminylphenol. Subsequently, we have synthesized N-acetyl and N-propionyl derivatives of cysteaminylphenol, NAcCAP and NPrCAP. NPrCAP was synthesised based on our expectation that the increased lipophilicity of the drug will improve the potency of the drug's toxicity. All of these synthetic compounds were found to be much better substrates for tyrosinase by having a higher affinity to tyrosinase but a slower reaction with tyrosinase-mediated oxidation. Since these synthetic compounds are good substrates for tyrosinase, we expected that they would result in increased cytotoxicity to melanoma cells and thereby provide the basis for development of novel anti-melanoma agents (Fig. 4).

The selective cytotoxicity of our synthetic compounds was screened by *in vitro* IC50 using MTT and colony forming assays. SKmel 23 and MeWo cells are highly melanized human melanoma cell lines, having very high tyrosinase activity. G361 is another type of melanotic melanoma cell line, but it has a lower tyrosinase activity. C32 and SKmel 24 are amelanotic human melanoma cells, having no tyrosinase activity. We have chosen fibroblasts and HeLa cells as control cells of the non-melanoma cell line. Cells were treated with NAcCAP or NPrCAP for 96 hours. All of the human melanoma cells showed much lower IC50 and much higher drug sensitivity as compared to control human fibroblasts. HeLa cells, however, also revealed increased sensitivity to our drug treatment. The cytotoxic effect of NAcCAP and NPrCAP was further confirmed by the colony formation assay. The two drugs revealed marked cytotocidal effect from the concentration of 0.2 mmol.

Following our initial screening of drug toxicity by *in vitro* MTT and colony formation assays, we evaluated the inhibition of DNA synthesis by NAcCAP on selected cell lines such as fibroblasts, HeLa cells, C32 and SKmel 23 human melanoma cells. SKmel 23 revealed increased drug sensitivity for inhibition of DNA synthesis. Thymidine incorporation study clearly showed that only melanoma cell lines of SKmel 23 and C32 revealed irreversible damage of DNA synthesis while a non-melanoma cell line of HeLa cells showed only transient reversible cytotoxicity, indicating both cytostatic and cytocidal effects of NAcCAP. Similarly the time dependent DNA synthesis inhibition by NAcCAP was found in SKmel 23 and C32 melanoma cells, but not in HeLa cells. NPrCAP also showed selective melanoma cytotoxicity with drug concentration dependent manner as can be shown with doses of 0.25, 0.50 and 1.00mM NPrCAP on SKmel 23 cells. In addition the comparison of viable cells after 5-day incubation of NPrCAP showed the cytotocidal effect of NPrCAP to melanoma cells of SKmel 23 and non-cytocidal effect of NPrCAP to non-melanoma cells of HeLa. These findings indicate that the drug effect of NAcCAP and NPrCAP, in particular NPrCAP, to non-melanoma cell lines is only cytostatic and transient, whereas it is cytocidal and permanent to melanoma cell lines. The selectivity and specificity of our synthetic compounds to melanoma cells can be shown in the following *in vivo* and *in vitro* studies.

The selective uptake of ^{14}C-labelled NAcCAP by melanoma and non-melanoma cells and tissues revealed clearly that a highly melanotic cell line of SKmel 23 takes the highest uptake of NAcCAP as compared to amelanotic melanoma cells of C32 and non-melanoma cell lines of HeLa and A431 cells. This uptake of NAcCAP is specific because if we added cold NAcCAP, there was a remarkable reduction of hot NAcCAP uptake by SKmel 23 cells. The specificity and selectivity of our drugs was further examined in the *in vivo* study, in which ^{14}C-NAcCAP was given *i.p.* to melanoma-bearing mice. Melanoma tissue of subcutaneous metastasis showed the highest incorporation and covalent binding whereas practically none of the uptake and covalent binding was seen in liver and kidney. In addition, a melanoma-bearing mouse showed, on the whole body autoradiogram, the selective uptake of NAcCAP in melanoma tissues of lung and skin.

We then examined to what extent one can block the *in vivo* and *in vitro* melanoma growth by administration of NAcCAP combined with BSO, buthionine sulfoxide. BSO that blocks the effect of anti-oxidants, was com-

bined in our chemotherapeutic regimen because the cytotoxicity of our NAcCAP is based upon the cytotoxic free radials and this cytotoxicity can be further increased by BSO. We found the combination of BSO and NAcCAP possess remarkable and synergistic antimelanoma effect in both *in vitro* culture cells and *in vivo* lung metastasis assays[21-24].

3 SUMMARY AND PERSPECTIVES

Four major topics of our current research efforts are introduced with the hope of establishing novel diagnostic and therapeutic approaches for malignant melanoma by exploiting our knowledge of basic molecular biology in melanogenesis. One of promising projects from our current research efforts is the development of a novel melanoma nano-medicine. The selective uptake and cytotoxicity of NAcCAP and NPrCAP may further be utilized for developing a novel drug delivery and anti-melanoma system. We are in the process of developing a combined hyperthermia and immuno-chemotherapy nano-medicine by conjugating NPrCAP with magnetite/liposome complexes. In this system NPrCAP can be employed as a novel drug delivery system and anti-tumor agent for primary and metastatic melanoma lesions by providing the selective uptake and toxicity to melanoma and by generating hyperthermic heat shock for immunotherapy. Interestingly if magnetite/cationic liposome complex was given to a subcutaneously grown melanoma nodule and then heat shock was generated to the whole body of experimental mice by an alternating magnetic field, there was regression of not only magnetite/cationic liposome injected melanoma nodule but also non-magnetite injected melanoma nodule. Further study indicated that this regression of non-magnetite injected melanoma nodule is derived from HSP-mediated immune reaction[25].

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