REVIEW

Current Update and Trends in Melanin Pigmentation and Melanin Biology

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Abstract. Certain aspects of current progress in melanin biology and its possible clinical relevances are reviewed with emphasis on some of our recent research activities. The important aspects discussed are (a) the biological properties of melanin pigments and their relevance to biological functions, (b) functional interaction of melanogenic/melanogenesis-associated genes in melanin biosynthesis process (melanogenesis, and (c) the targeting of proteins involved in melanogenesis and assembly by melanosomal proteins. Upon exposure to UV light radiation (UVR), melanin pigments revealed two distinct photobiological reactions, *i.e.* photoprotective and phototoxic reactions. The precursor intermediate of brown-black eumelanin, 5-6-dihydroxyindole, appears to possess the most potent photoprotective (antioxidant) property. Eumelanin pigment also had some antioxidant property. Similarly, yellow-red pheomelanin and its precursor intermediate, 5-S-cysteinyl-dopa also revealed some antioxidant property, but they became prooxidant in the presence of the ferric iron upon exposure to UVR. Melanosomes are known to possess several metal ions including Fe^{2+} , Fe^{3+} , Cu^{2+} and Zn^{2+} . In addition, upon exposure to UV-light, there is an increase in ferric/ferrous iron in the skin. Therefore, in the *in vivo* system, pheomelanin intermediate, 5-S-cysteinyl-dopa, may show significant prooxidant property in conjunction with metal ions (e.g. Fe^{2+} , Fe^{3+}). When atypical moles (previously called dysplastic nevi) were analysed chemically for quantitative and qualitative properties of melanin pigment, they revealed a high ratio of pheomelanin/eumelanin content. This finding may partly explain our clinical observation that these moles are frequently the precursors of malignant melanoma and that the intermittent heavy exposure of UVR can be the major direct cause of their transformation. How, then, the melanosomal compartment, where active new melanin synthesis occurs after exposure to UVR, is protected from the cytotoxicity of melanin precursor intermediates. To study this question, two major experiments were conducted; (a) expression of melanogenesis-associated genes upon exposure of melanocytes to UVR and (b) transfection of cDNAs from the melanogenesis-associated genes. There was coordinated gene expression (mRNA) of tyrosinase and tyrosinase-related protein, TRP-1 and this coordinated gene expression was also accompanied by the upregulation of LAMP-1 (lysosome-associated membrane protein-1). Furthermore, human tyrosinase and TRP-1 mRNAs were expressed successfully in individual transfectants or co-transfectants of cDNAs from the two genes. Co-transfectants of human tyrosinase and TRP-1 cDNAs produced many lysosomal granules and melanin-containing granules, melanosomes. LAMP-1 gene was upregulated simultaneously in co-transfectants of tyrosinase and TRP-1, but not in individual transfectants of the two genes. TRP-1 and LAMP-1 gene products were transferred from Golgi complexes to melanosomes. LAMP-1 gene products stimulated by TRP-1 and tyrosinase genes may prevent programmed death of melanocytes due to cytotoxic melanin precursor intermediates by coating the inner surface of melanosomal membrane through the N- and O-linked oligosaccharide moieties of LAMP-1 which have been suggested to protect lysosomes from the action of hydrolytic enzymes. (Keio J Med 44 (1): 9–18, March 1995)

Key words: melanogenesis, melanocyte, malignant melanoma, UV light

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Abbreviations: UVR; ultraviolet light radiation, TRP; tyrosinase-related protein, DHI; dihydroxyindole, DHICA; dihydroxyindole-2-carboxylic acid, EPR; electron paramagnetic resonance, SCD; 5-S-cysteinyl-dopa, TMB; tetramethyl benzidine, MDA; malondialdehyde, TBARS; thiobarbituric acid reactive substances, LAMP; lysosome-associated membrane protein, HMSA; human melanosome specific antigen.

Introduction

Human melanocytes migrate from the embryonic neural-crest tissue into the skin and then produce unique secretory granules, melanosomes, which contain melanin pigments. Melanin and melanosomes are the major determinants of skin pigmentation. Melanin-forming enzyme tyrosinase is the major component responsible for melanogenesis, which occurs within the compartment of secretory granules, melanosomes.¹

Mechanisms involved in the biosynthesis and structural organization of melanosomes are not well understood. An elucidation of the molecular events involved in these processes would be important in understanding the biology and pathophysiology of normal and abnormal pigmentation in the skin. The pathological role of pigmentation originates mainly from the fact that melanin pigments serve not only as the major determinant of the skin color but also as the major source of skin protectant against radiation, preventing sun-induced skin damage as well as skin cancer development.² This elucidation may be equally important for an explanation as to why the melanosomal structures become markedly aberrant in various melanocytic disorders including neoplastic counterpart, malignant melanoma.³ Abnormal melanin pigmentation can also be the direct cause of a number of congenital and acquired pigmentary skin diseases.⁴ For example, abnormal degradation of melanosomes has been suggested to be a direct cause of programmed death of melanocytes of vitiligo and related diseases.⁵ These changes may result from abnormal expression of genes involved in the biosynthesis of melanin and melanosomes, which leads directly or indirectly to alteration in the structure and/or function of melanosomes. Disturbed rates of transcription and putative alternative splicing of mRNAs may also contribute to the melanosomal derangement which would lead to the formation of abnormal melanosomes seen in such diseases as von Recklinghausen's disease and Chediak-Higashi syndrome.⁶ This review introduces three major topics. The first topic deals with biological properties of melanin pigments, in particular, after exposure to UVR, and the second one addresses the functional interaction of tyrosinase and LAMP gene families in melanogenesis, using the cells exposed to UVR. Signals targeting the transport of proteins into the melanosomal compartment may be disturbed due to altered co- and/or post-translational modification. A better understanding of such putative biological and physiological processes may

best be obtained by isolating DNA probes for genes relevant to melanogenesis, transfecting these genes in either wild or mutated form into non-pigmented or amelanotic cells and correlating the expression of mRNAs and their products with cell growth and differentiation. The third topic, therefore, is the possibility of using the gene transfection experiments in understanding the target signals of melanogenic proteins and melanosomal assembly.

Biological Properties of Melanin Pigments

UV radiation and melanin pigmentation

UV light can be the most potent stimulator for growth and differentiation of melanocytes. Melanocyte activation and differentiation can be induced directly by UV irradiation or indirectly through the interaction with UV-irradiated surrounding keratinocytes. For example, UV radiation can increase the synthesis of bFGF in keratinocytes which will then stimulate the melanogenesis of epidermal melanocytes.^{7,8} Similarly, MSH production by keratinocytes can be stimulated by UV radiation and also enhance the expression of MSH receptor on the melanocyte which will then increase the melanogenesis by the melanocyte.⁹ There are, of course, growth factors and regulators which are not directly related to UV exposure. For example, c-Kit which is a proto-oncogene encoding a membrane receptor protein of molecular weight of 145 kDa with intrinsic tyrosine kinase activity in melanocytes. C-Kit gene expression is essential for the proper embryonic development of melanocytes (Fig 1).¹⁰ If there is any mutation of c-Kit, this could cause white spotting in mice and piebaldism in humans, affecting the proliferation and survival of melanocyte precursors during embryonic differentiation. The c-Kit expression would also be important in the proliferation of adult melanocytes and may also be partly related to their differentiation after exposure to UVR.

The melanocyte performs the unique metabolic activity of melanin synthesis. In a complex series of reactions occurring in specialized organelles, melanosomes, tyrosinase catalyzes the first two reactions of the biochemical pathway leading to the formation of two types of highly polymerized melanin pigments, eumelanin or pheomelanin (Fig 2). Tyrosinase catalyzes the hydroxylation of tyrosine to form dopa (L-3,4-dihydroxyphenylalanine) and then the oxidation of dopa to dopaquinone. The latter is rapidly converted to the brown-black pigment, eumelanin, via 5,6-dihydroxyindole (DHI) or dihydroxyindole-2-carboxylic acid (DHICA). However, intervention of sulphhydryl compounds in melanogenesis leads, via 5-S-cysteinyl-dopa (SCD), to the formation of the yellow-red pigment, pheomelanin (Fig 3). Eumelanin is the major pigment in normal human skin, although small

amounts of pheomelanin can be seen in certain ethnic groups (e.g. red-haired, freckled Caucasians). The exposure of human melanocytes to UV light or melanocyte

stimulating hormone increases the production of melanosomes as well as eumelanin pigments. EPR (Electron Paramagnetic Resonance) studies have provided evidence for the presence of semiquinone radicals ($QH^{\bullet-}/Q^{\bullet-}$) in eumelanin¹¹ and our own studies have shown that the

Fig 1 Growth factors and regulation in cascade of melanocyte differentiation and transformation UV can be the most potent stimulator for melanogenesis. It can stimulate the production of MSH (melanocyte stimulating hormone) in the keratinocyte and upregulate the MSH-receptor on the melanocyte, which would then be stimulated to produce new melanin pigment through cyclic AMP cascade. It can also affect the transformation of melanocytes and dysplastic melanocyte (DMC) which would then follow the step-wise growth phases from radical growth phase (RGP) to vertical growth phase (VGP) and finally metastatic growth phase (MGP). Fibroblasts (FB) in the dermis can also produce the melanocyte differentiation and growth factor (e.g. basic fibroblast growth factor: bFGF).

Fig 2 Metabolic and chemical reactions of eumelanin synthesis.

Fig 3 Metabolic and chemical reactions of pheomelanin synthesis.

concentration of the semiquinone radicals increases on irradiation ($>300\text{nm}$).² While it is not clear whether these melanin radicals are cytotoxic or not, they could participate in known physiologically harmful free radical reactions (Fig 4).

Melanins have been shown to produce $\text{O}_2^{\cdot-}$ on irradiation.¹² Excessive production of $\text{O}_2^{\cdot-}$, H_2O_2 , $\text{OH}^{\cdot-}$ and Fe^{2+} could lead to cell damage. However, the contribution of these reactions to cytotoxicity is likely to be minimal because these reactions are very slow at acidic pH and melanosomal pH is in the range of 3.0–4.5.¹³ Despite this, there are reports showing cytotoxic effects for eumelanin and pheomelanin.¹⁴ In these experiments, melanin added to the cell culture medium and taken up by the cell might end up in the cytosol and other subcellular compartments where conditions (pH) are likely to be more favourable for the above damaging free radical reactions.

Fig 4 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-cysteinylDOPA 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.

Photoprotective vs phototoxic actions of melanin pigments

In order to study the photoprotective and phototoxic actions of melanin pigments, the experimental conditions mimicking melanosomes were prepared artificially by using soy bean phosphatidylcholine liposomes. The lipid peroxidation in UV-irradiated liposomes were measured as a means to examine the primary membrane events after exposure of melanins and their precursors to UV light.^{15,16} Liposomes were made from phospholipids containing unsaturated fatty acyl chains which are vulnerable to oxidative damage. Two stages of lipid peroxidation were measured in this study. The early lipid peroxidation stage, conjugated dienes by UV absorption and the production of hydroperoxides by iodometry and tetramethylbenzidine (TMB), and the late lipid peroxidation stage, formation of malondialdehyde (MDA) by HPLC and thiobarbituric acid reactive substances (TBARS) by spectrophotometry were examined in the presence of various sources of melanins, including eumelanin and DHI as well as pheomelanin and SCD.

Both eumelanin and pheomelanin were effective in decreasing UVR-induced lipid peroxidation product, with pheomelanin being more effective than eumelanin for all three methods of detection, *i.e.* conjugated dienes, iodometry and TMB. Our preliminary experiment measuring lipid peroxidation has also shown that natural squid eumelanins have anti-lipid peroxidation properties similar to those of three different synthetic eumelanins made from dopa oxidation. The greater anti-oxidant activity of pheomelanin over eumelanin may partly be a reflection of the fact that the former is completely dissolved while the latter remains as a suspension during irradiation. The melanin precursors, DHI and SCD, were even more effective than the mature pigments, DHI having more significant antioxidant activity than SCD.¹⁷

The decrease in lipid peroxidation occurred, not only in the early stage of lipid peroxidation product such as conjugated dienes and lipid hydroperoxide, the formation of which might be rapidly reversible *in vivo* and *in vitro*, but also in the potentially more carcinogenic late-stage product such as TBARS. Pheomelanin was an effective antioxidant at concentrations as low as 5 µg/ml. In contrast, eumelanin showed no antioxidant activity when late-stage products were measured. Eumelanin precursor, DHI, showed the highest antioxidant property in late-stage lipid peroxidation. The ability of pheomelanin, its precursor, SCD, and eumelanin precursor, DHI, to decrease the formation of late-stage lipid peroxidation product is an indication of their participation in preventing the initiating species or the breaking of chain reaction products involved in lipid peroxidation. The TBARS method is applicable in the presence of iron and this has allowed us to characterize some potentially harmful

properties of melanin and their precursors. The addition of a comparatively high concentration of ferric iron during UVR exposure, we changed the oxidative environment of the pigment and lipids. Ferric iron is known to be present within the melanosomal compartment. Metal iron and its ionic forms are also known to increase in keratinocytes after UVR exposure.^{18,19} The presence of ferric iron caused the mature pigment to switch from functioning as an antioxidant membrane protector to membrane damaging pro-oxidant when exposed to UVR. This effect was reversed in the presence of EDTA which chelates the iron. In the presence of ferric iron at low concentration, SCD also showed the highest pro-oxidant effect. Importantly, however, DHI did not show any pro-oxidant activity at both high and low concentrations of ferric iron and remained as an effective antioxidant. Under pro-oxidant conditions of all tested pigments, pheomelanin was found to be the most potent inducer of late-stage peroxidation product. One can, therefore, speculate that membrane-damaging potential of pheomelanin and SCD under pro-oxidative conditions would make them hazardous if these conditions arise *in vivo* such as after heavy intermittent exposure to UVR during summer.

Perturbed melanin synthesis and increased UVR sensitivity in possible melanoma precursor lesions: Familial atypical multiple moles and melanoma syndrome

To further evaluate the possible functional role of melanin pigmentation, the amount of eumelanin and pheomelanin was directly measured in the tissue of dysplastic melanocytic nevi (or atypical moles) because patients suffering from familial atypical multiple moles and melanoma (FAMMM) or previously called familial dysplastic melanocytic nevus syndrome possess a high risk for melanoma development as much as 80% to 100% in their lifetime.^{20,21} In these syndrome patients, there is an increased susceptibility to UV-induced somatic mutation such as enhanced cell killing upon UV exposure or altered DNA repair and the abnormal DNA synthesis which is, however, distinct from that of XP variant cell lines.²²

The pheomelanin content of common melanocytic nevi was compared to atypical moles (previously called dysplastic melanocytic nevi).²³ The data clearly showed an increase in the amount of pheomelanin in dysplastic melanocytic nevi as measured by AHP, a pheomelanin indicator, and PTCA, a eumelanin indicator.

The direct measurement of tissue content of eumelanin and pheomelanin in atypical moles indicates that there may be perturbed melanogenesis in patients with this syndrome. Furthermore, it was shown that melanosomes in atypical nevocytes were oval in shape and contained granular material similar to that seen with pheomelano-

somes.²⁴ The recent study from several research groups also indicated that there is an increased level of pheomelanin precursor, SCD in these atypical moles and there is an increased blood level of SCD as well as other melanin metabolites after UVR in melanoma patients.²⁵⁻²⁷ All these findings indicate that pheomelanin and its precursor, SCD, in the presence of UVR may be harmful in certain patients such as FAMMM syndrome patients who have a high risk of melanoma development.

Melanogenesis and Functional Interaction of Tyrosinase and Lamp Gene Families

Major genes and their products involved in melanogenesis cascade

Three genetic loci are of the greatest importance in the melanin synthesis pathway, and most extensively studied in laboratory mice. These are the *c* (color)-locus for tyrosinase, the *a* (agouti)-locus for amounts and distribution of pheomelanin and eumelanin within the integument, and the *b* (brown)-locus for the type of eumelanin synthesized by melanocytes.^{28,29} The level of tyrosinase mRNA and tyrosinase is elevated in brown (*b/b*) melanocytes suggesting an influence of the *b* locus on the transcription of the tyrosinase gene.³⁰ The *b*-locus deletions or null point mutations do not eliminate pigmentation, but result in mice with brown, instead of black eumelanin.³¹ The *b*-locus also codes for a protein forming part of the melanosomal matrix. This *b*-locus protein possesses 36% amino acid homology to tyrosinase, and has been termed tyrosinase-related protein-1 (TRP-1). Other mutations at this locus result in an intermediate phenotype between black and brown, due to a reduction in the amount of TRP-1 mRNA, whilst still others have a dominant phenotype resulting in premature melanocyte death.³² This gene has been initially isolated as a candidate gene for tyrosinase by immunoscreening of a melanocytes cDNA expression library using anti-tyrosinase antibody (TMH-1). When the same melanocyte cDNA library was immunoscreened by the same anti-tyrosinase antibody, another cDNA was identified. This clone showed some cross hybridization with a TRP-1 cDNA, but encoded a different protein (hence called TRP-2). The protein was found to have a dopachrome tautomerase activity and its human cDNA has been cloned recently³³ and mapped to mouse *s* (slaty-slt) locus.^{38,41}

Functional interaction of tyrosinase and LAMP gene families after exposure to UVR

Human tyrosinase and LAMP-gene families share many common structural and biological properties. In fact, melanosomes and lysosomes are known to possess

many similarities and could derive from common precursor vacuoles (Fig 5). Human LAMP-1 and LAMP-2 sequences reveal strong homology between the two molecules, particularly in the proximity to the COOH-cytoplasmic terminal end. It is suggested that this portion is important for targeting the molecules to lysosomes.^{34,35} Similar to the LAMP molecule, tyrosinase and TRPs possess a single transmembrane domain, and the cytoplasmic tails at COOH terminal show some common features such as motif with basic amino acids and putative tyrosine phosphorylation sites.^{36,37} These structural characteristics may contribute to the activation of melanogenesis-associated proteins by allowing them to target to the melanosomal membrane, and this targeting signal could be regulated by the tyrosine phosphorylation-dephosphorylation of cytoplasmic tails. Importantly, tyrosinase and TRP-1, possesses two putative tyrosine phosphorylation sites which are common, whereas LAMP-1, LAMP-2 and TRP-2 show only one putative tyrosine phosphorylation site, but this site is identical or similar to one of the two putative tyrosine phosphorylation sites seen in tyrosinase and TRP-1. Therefore, there may be some common functional properties between tyrosinase and LAMP-gene families or some structural interaction between these two melanogenesis-associated gene families. Human melanoma cells and normal melanocytes increase the expression of tyrosinase and Lamp-gene families and their gene products after exposure to UVR.³⁸

The findings of increased melanin synthesis in pigmented melanoma cell lines and the absence of melanin synthesis in amelanotic melanoma cells after exposure to UVR were in parallel to those findings of semi-quantitative RT-PCR analysis of mRNA expression of tyrosinase, TRP-1, LAMP-1 and LAMP-3 in these melanotic and

Fig 5 Expression of tyrosinase and LAMP gene family products in melanosomes and lysosomes. LAMP gene family is expressed in both the melanosome and lysosome.

amelanotic cell lines after exposure to UVB for seven consecutive days. There is an increased expression of tyrosinase in pigmented melanoma cells, e.g. G361 which is followed by an increased expression of TRP-1. There is no tyrosinase gene expression in amelanotic melanoma cells, e.g. SK-MEL-24 before and after UVB exposure, which is again followed by the negative expression of TRP-1 in the same cell line. Interestingly, certain amelanotic melanoma cell lines, e.g. C32 showed the presence of increased tyrosinase gene expression which was, however, not associated with TRP-1 expression. It appears, therefore, that the proper melanin biosynthesis within the melanocyte may require the coordinated mRNA expression of both tyrosinase and TRP-1. In contrast, there was no alteration in the expression of LAMP-3 which appears to be more related to the structural component of the lysosomal or melanosomal membrane.

Functional interaction of tyrosinase and LAMP gene families after tyrosinase gene family transfection

In order to further study the functional interaction of LAMP and tyrosinase-gene families in melanogenesis, three major experiments have been carried out recently by our research group. These include; (a) co-transfection of tyrosinase and TRP-1 into amelanotic melanoma cells, (b) treatment of melanotic melanoma cells with antisense oligodeoxynucleotides against LAMP-1, and (c) sub-cellular distribution of tyrosinase/TRP-1 and LAMP-1/LAMP-3.

For the co-transfection of human tyrosinase and TRP-1 genes, human amelanotic melanoma cells of SK-MEL-24 and C32 as well as COS7 monkey kidney cells were used. C32 has tyrosinase gene expression but there is no actual melanin synthesis and no TRP-1 gene expression. In contrast, SK-MEL-24 lacks both tyrosinase and TRP-1 gene expression. After co-transfection of human tyrosinase cDNA, there was an increased tyrosinase activity as well as melanin synthesis. In contrast, TRP-1 transfection did not show any such increase of tyrosinase activity and melanin synthesis, indicating that TRP-1 itself is not responsible for direct activation or synthesis of tyrosinase as well as melanin pigments. However, it may help tyrosinase-transfected cells to protect themselves from programmed cell death due to tyrosinase-mediated toxicity. This hypothesis was further substantiated by our finding that melanoma cells and COS-7 cells transfected with human tyrosinase gene alone revealed many vacuolations, and even premature death of cells, indicating that human tyrosinase gene product itself is toxic to the cells. In contrast, co-transfection with human tyrosinase and TRP-1 genes showed the production of many oval granules which were positive with anti-TRP-1 antibody immunostaining and contained melanized melanosomal matrix under electron microscopic observation. There

was no vacuolation as was seen in cells with single transfection of human tyrosinase gene. TRP-1 transfection alone did not influence directly any melanin synthesis. There was no increase of tyrosinase nor was there any increase of melanin synthesis.^{39,40}

To further investigate the functional interaction between tyrosinase and LAMP-family genes, comparative PCR analyses were carried out using specific primers targeting Lamp-family genes, including LAMP-1, LAMP-2 and LAMP-3 as well as tyrosinase and TRP-1 cDNAs. Expression of LAMP-3 was detected in all cells treated with either individual transfection of human tyrosinase or TRP-1 gene or co-transfection of both. No significant variations were observed among the individual experimental groups. In contrast, enhanced mRNA expression of LAMP-1 gene was observed in human tyrosinase and TRP-1 co-transfected cells, but not in individual transfectants. These observations were shown by the protein expression study using Western blotting. The results may indicate that there is a coordinated up-regulation of LAMP-1 gene and protein synthesis by co-transfection of tyrosinase and TRP-1 cDNAs, but not by a single transfection of either one of them. This hypothesis was further tested by the antisense LAMP-1 gene hybridization experiment. In this experiment, human melanoma cells of G361 were incubated with antisense oligodeoxynucleotide targeted on LAMP-1 gene in culture. They were then processed for immunoprecipitation using 3 different monoclonal antibodies against human TRP-1 such as HMSA-5, 3B5 and 2G10. This antisense experiment has clearly shown significant and coordinated decrease in the expression of TRP-1 in G361 melanoma cells which were hybridized with anti-LAMP-1 oligodeoxynucleotide. TRP-1 is an N-linked glycoprotein, having approximately 6 to 8 oligosaccharides after N-glycanase digestion. Therefore, human TRP-1 which was originally 69kDa in size moved approximately to 50kDa after N-glycanase digestion. TRP-1 does not, however, have any O-linked oligosaccharides.

In order to characterize the glycosylation process of human TRP-1, cells were treated with tunicamycin which inhibits all the processes of glycosylation and monensine which inhibits the glycosylation process at the site of medial portion of the Golgi complex. In cultured human cells such as normal human melanocytes and melanoma cells treated with monensine, there was a remarkable increase in the expression of TRP-1 as compared to control. In contrast, almost complete loss of TRP-1 expression was seen after treatment of tunicamycin (Fig 6).⁴¹ The findings clearly indicated that TRP-1 is transferred from ER to the medial portion of the Golgi complex where full glycosylation may be completed. On the other hand, tyrosinase will be processed during glycosylation to the periphery of the Golgi network, trans-Golgi network, and then subsequently transferred

Fig 6 Coordinated interaction of tyrosinase (Ty) TRP-1 and LAMP-1. Inhibition of glycosylation of tyrosinase and TRP-1 by tunicamycin and monensin. When melanoma cells are exposed to monensin, which perturbs the transport of glycoproteins at the site of medial Golgi, there was an increased expression of TRP-1. Tunicamycin, which inhibits the glycosylation of glycoproteins at the site of ER (endoplasmic reticulum), inhibited completely the expression of TRP-1 and tyrosinase.

to the melanosomes.

Under electron microscopic immunogold study of HMSA-5, which is identical to TRP-1, immunogold particles were labelled on the medial portion of the Golgi complex as well as small vesicles but not coated vesicles in the vicinity of Golgi complexes.⁴² Importantly, the same immunogold positive vesicles were seen on the surface of secondary lysosomes. Specifically, HMSA-5 positive immunogold particles were seen along the outer membrane of the melanosomes. They were accumulated on the surface of melanosomal membrane in the early melanosomal maturation, such as in stages 1 and 2. The size of individual microvesicles or immunogold positive vesicles ranged approximately from 40 nm in diameter. These small vesicles were present in various stages of melanosomal maturation, and located along the surface of the melanosomal membrane. When they were present in mature stage 4 melanosomes, they often revealed the electronlucency. At this stage, HMSA-5 expression was negative because the antigenic epitope of HMSA-5 appeared to be embedded in the highly melanized melanosomal matrix.⁴²

Importance of glycosylation for proper melanization within melanosomal compartment

When immunoelectron microscopic study was done on immunogold-particle distribution reacted with anti-HMSA-7, which is relevant to LAMP-3 or CD63, and the immunogold particles were located, similar to HMSA-5, within the small vesicles, associated with melanosomal membrane. This was further confirmed by anti-LAMP-3 immunoelectron micrograph using LS62, indicating that LAMP-3 is present in both melanosomes

and lysosomes.

In contrast, tyrosinase is present in the trans-Golgi network and the coated vesicles and melanosomes in late stages of 2, 3, and 4. Importantly, there are a fairly large number of small vesicles present in the vicinity of Golgi complexes, but none of these small vesicles are positive with tyrosinase activity. The structure and distribution pattern of these small vesicles were identical to those of HMSA-5 (TRP-1) or HMSA-7 (LAMP-3) molecules. Immunoelectron microscopic and cytochemical studies indicate that TRP-1 and probably also LAMP gene family proteins, take the subcellular localization different from that of tyrosinase in the Golgi complex. One may then wonder why TRP-1 and LAMP gene families need to have the subcellular distribution different from that of tyrosinase before they are incorporated into melanosomes and what the functional interaction is present between TRP-1 and LAMP gene family during melanization.

This question may be better answered when one compares the structural properties of tyrosinase and LAMP gene family products. TRP-1 is an N-linked glycoprotein which has only 6 to 8 N-linked oligosaccharides but no O-linked oligosaccharides. In contrast, LAMP-1 and LAMP-2 are of similar molecular size and have both N-linked and O-linked oligosaccharides, indicating that LAMP-1 and LAMP-2, but not LAMP-3 are highly glycosylated as compared to TRP-1. It is known that in lysosomes, the LAMP-1 and LAMP-2 coat the inner surface of the lysosomes, serving as a barrier to prevent the action of lysosomal hydrolases on the membrane.

Similar to lysosomes, LAMP-1 and probably LAMP-2 molecules which are up-regulated after co-transfection of tyrosinase and TRP-1, are present on the inner surface of melanosomal membrane, functioning as a scavenger of free radicals and other free radical products which are produced during new melanogenesis through tyrosinase activation. This process, however, does not appear to be activated by either tyrosinase or TRP-1 alone. In other words, tyrosinase and TRP-1 coordinate together to up-regulate LAMP-1 which will then be transferred to the melanosomes, coating the inner surface of the melanosomal membrane. If this speculation were true, then LAMP-1, not TRP-1, is a key molecule which directly protects the melanosomal membrane against tyrosinase-mediated membrane damage, by having a high content of N- and O-linked oligosaccharides. In contrast, TRP-1 has only a small number of N-linked oligosaccharides and no O-linked oligosaccharides, so that it can hardly function as a barrier for tyrosinase-mediated oxidative stress within the melanosomal compartment (Fig 7).³⁷

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Fig 7 Possible functional interaction of tyrosinase and LAMP gene family products. Calnexin would be a commander of glycosylation of the intermediate molecules of these gene products. Upon the coordination of tyrosinase and TRP-1, LAMP-1 will be upregulated, coating the inner surface of melanosomal membrane which would protect the membrane damage of melanosomal compartment resulting from tyrosinase-mediated oxidation.

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