Biological and Biochemical Studies on Melanogenesis and Melanoma Cells

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ABSTRACT

For the previous ten years, we have been studying intracellular transport of melanosomal proteins and their biological and biochemical functions in melanoma cells. Melanosomal proteins, tyrosinase, tyrosinase-related protein 1 (TYRP1) and TYRP2/DOPAchrome tautomerase (DCT), gp100/Pmel17 are transported from the trans-Golgi network (TGN) to early-stage melanosomes via endosomal compartments. We suggest that Rab7 is involved in the vesicular transport of tyrosinase and TYRP1 and in the melanogenesis through the regulation of gp100/Pmel17 maturation. TYRP1 and TYRP 2/DCT were shown to play an essential role in suppressing TYR-mediated cytotoxicity in melanocytic cells, possibly through interaction with TYR in melanosomes.

We also studied apoptotic cell death of melanoma cells and death mediators. Among p53 family members, p51A (p63) induced apoptosis in both wild-type and mutant p53-expressing melanoma cells more significantly than p53 and p73β. Interferon (IFN) exerts anti-tumor activities possibly by regulating IFN-stimulated genes. Caspase-2 activation was commonly associated with induction of apoptosis in IFN-β-sensitive melanoma cells. The diacylglycerol kinase (DGK) α, expressed in several human melanoma cell lines but not in melanocytes, was a novel positive regulator of NF-κB, which suppresses TNF-α-induced melanoma cell apoptosis.

Key words : Melanogenesis, Melanin, Apoptosis, Melanoma cells

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Abbreviations : SSM; superficial spreading melanoma, ALM; acral lentiginous melanoma, NM; nodular melanoma, LMM; lentigo maligna melanoma, UV; ultra violet light, ROS; reactive oxygen species, TYRP1; tyrosinase-related protein 1, DCT; DOPAchrome tautomerase, IFN-β; interferon-β, TNF-α; tumor necrosis factor-α, TGN; trans-Golgi network, ISGs; IFN-stimulated genes, TRAIL; tumor necrosis factor-related apoptosis-inducing ligand, NF-κB; nuclear factor-κB, DGK; diacylglycerol kinase

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INTRODUCTION

The majority of melanoma cases derive from melanocytes in the skin, but small numbers also appear in the mucosal membranes, eye and the central nervous system. The number of patients suffering from melanoma gradually increased in Japan from 1987 through 2001, particularly those afflicted with the subtype of superficial spreading melanoma (SSM) [1]. The 140-month survival rates for females and males with Japanese melanoma were 70.6% and 60 %, respectively[1]. A survey was conducted in 2006 and 2007, in which 1,053 cases of malignant melanoma from 160 institutions in Japan were examined (male/female ratio: 520/532; average age: 62.1 years). According to the survey, among Clark's subtypes, acral lentiginous melanoma (ALM) occurred most frequently (51.2%), followed by nodular melanoma (NM, 21.2%), SSM (16.2%) and lentigo maligna melanoma (LMM, 7.9%) [2]. In the past ten years, the employment of sentinel lymph node biopsy using RI and dye has spread among the major institutions in Japan, resulting in a decrease in the number of patients with lymphedema accompanied with complete lymph node dissection. Although early lesions of primary melanoma, namely melanoma *in situ* and malignant melanoma with tumor thickness less than 1.0 mm, are curable by exci-

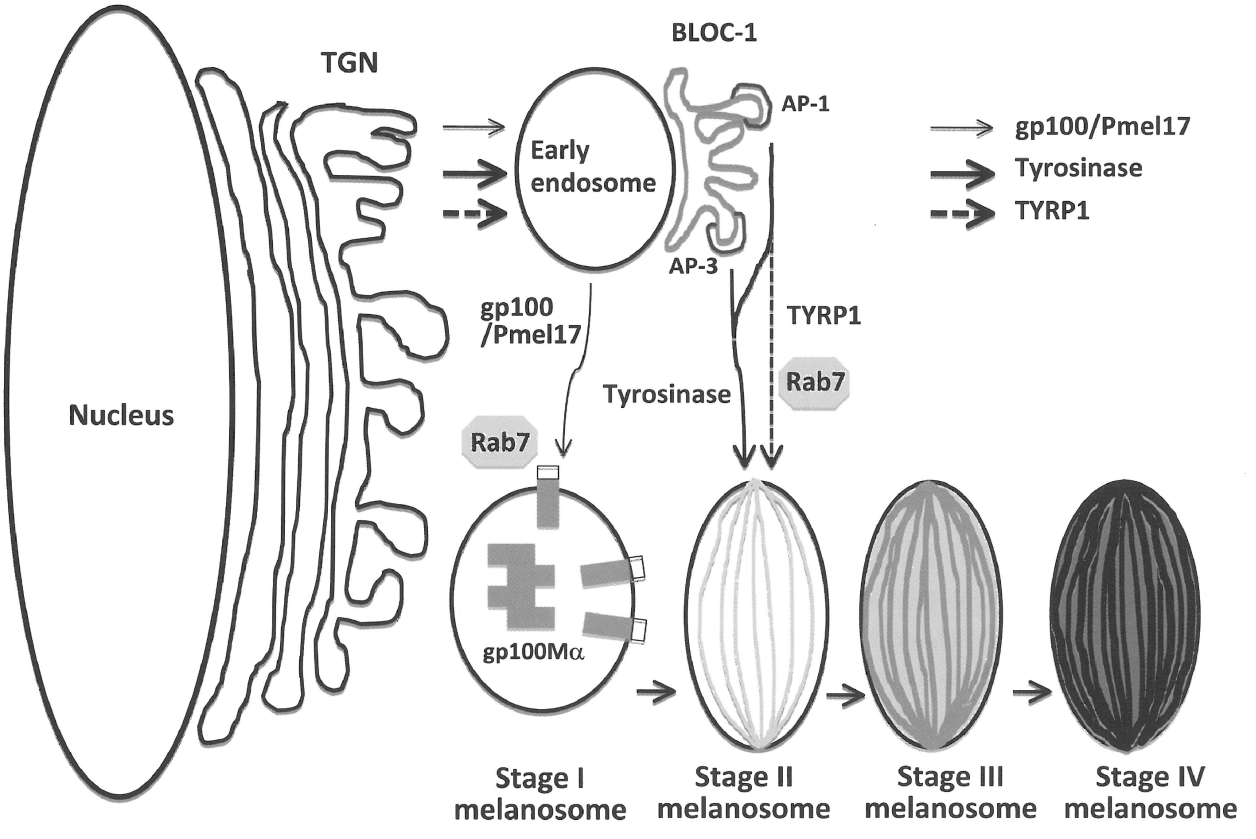

Figure 1 Vesicular transports of melanosomal proteins and formation of melanosomes.
Melanosomal proteins, tyrosinase, TYRP-1 and Pmel17/gp100, are transported to early endosomes. Pmel17/gp100 is then processed to generate N-terminal Mα to form fibrillar amyloid sheets. Tyrosinase and TYRP-1 are transported to stage II melanosomes through BLOC-1/AP-3 and BLC1/AP-1, respectively[5]. Rab7 accelerates the maturation of and accumulation of Pmel17/gp100 Mα fragments to form stage I melanosomes. In the transport of tyrosinase and TYRP-1, Rab7 has some roles to enhance their move from early endosomes to stage II melanosomes. In details see text.

sion, successful treatment of metastatic melanoma has been elusive thus far.

To develop melanoma-specific targeting and therapy, it is essential to elucidate biological and biochemical characterization of melanocytes and melanoma cells including melanogenesis, melanosome-associated proteins, apoptotic cell death and signal transduction pathways activated or suppressed. In this review, we summarize our previous papers regarding the melanogenic proteins and apoptotic cell death of melanoma cells. Melanin is synthesized and sequestered within the membranous compartment in the cytoplasm of melanocytes called melanosomes. Melanosome-resident proteins are expressed only in melanocytes and most of them are integral membrane proteins, such as tyrosinase, tyrosinase-related protein 1 (TYRP1), TYRP2 or DOPAchrome tautomerase (TYRP2/DCT) and gp100 or Pmel17 (gp100/Pmel17) [3),4)]. Melanosomes are considered to be lysosome-related organelles derived from early endosomal membranes [5)]. However, a molecular basis of vesicular transport of the melanosome proteins and melanosome formation has not yet been elucidated.

Since induction of apoptosis in melanoma cells will provide important clues for effective therapy against malignant melanoma, we studied apoptotic cell death mediated by p53 family members, interferon-β (IFN-β) and tumor necrosis factor-α (TNF-α). Since melanogenesis and the apoptotic pathway may be shared among various types of melanomas despite the different gene mutations and activated pathways, this research may contribute to the clinical treatment and management of melanomas.

1. Vesicular transport of melanogenic proteins and melanosome formation

(1) Tyrosinase and TYRP1

Melanosome biogenesis consists of multistep processes that involve the synthesis of melanosomal proteins followed by their vesicular transport and post-translational modifications such as glycosylation, proteolysis and oligomerization. Tyrosinase, a critical enzyme for melanogenesis, and TYRP1, functioning as 5, 6-dihydroxyindole-2-carboxylic acid oxidase in mice, are transported from the trans-Golgi network (TGN) to early-stage melanosomes via endosomal compartments [3)]. It has been reported that gp100/Pmel17, a melanosomal structural protein, is also transported from TGN to early-stage melanosomes [6)]. They pass through different pathways consisting of different molecules; tyrosinase and TYRP1 are transported from early BLOC-1-positive endosomal structures with AP-1 or AP-3 to stage II melanosomes, while gp100/Pmel17 are moved to stage I melanosomes via early endosomes [5),7)](Fig. 1). We showed the experimental findings suggesting Rab7, low-molecular-weight GTP-binding protein, is involved in the vesicular transport of tyrosinase and TYRP1, and in the maturation of gp100/Pmel17.

Rab7 is a member of the Rab small GTP-binding protein and essential for the regulation of endosomal/lysosomal vesicular transport and phagocytosis [8)]. Previously we have reported that Rab7 is localized on the melanosomal membrane of B16 murine melanoma cells [9)]. The cells treated with Rab7 antisense oligonucleotide revealed TYRP1 was confined to the perinuclear area, suggesting that Rab7 is involved in the transport of TYRP1 from TGN to melanosomes, possibly passing through late endosomes. Exogenous expression of TYRP1 and Rab7N125I, a dominant negative mutant of Rab7, in the amelanotic melanoma cells revealed TYRP1 to be localized to early endosomes, suggesting that the transport of TYRP1 may require functional Rab7 [10)](Fig. 1).

Immortal mouse melanocytes were transfected with plasmids carrying cDNA of wild-type and mutant Rab7s to analyze their differential effects on the intracellular trafficking of tyrosinase, TYRP1 and gp100/Pmel17. As a result, the inhibition of the Rab7 function resulted in preferential TYRP1 elimination possibly by cellular degradation machinery. It has been suggested that the vesicular transport pathway of

TYRP1 from TGN to melanosomes is different from that of tyrosinase and gp100, and that Rab 7 is a crucial regulator for TYRP1 transport in melanogenesis cascade [11].

(2) gp100/Pmel17

gp100/Pmel17 is cleaved to its functional Mα and Mβ fragments after being transported from the TGN to the stage I melanosomes. The N-terminal Mα fragments serve to form the fibrillar matrix of the organelle [12),13] In the subsequent stages of maturation and eumelanogenesis, the fibrillar amyloid sheets of Mα fragments allow the organelle to develop an ellipsoidal shape characteristic of the stage II melanosomes [13](Fig. 1). Melanins are deposited on these fibrillae, resulting in a progressively pigmented internal matrix (stage III). Melanin synthesis and deposition continue until little or no internal structure remains visible (stage IV). However, the precise mechanism of melanosome biogenesis is still unclear. We studied the molecular mechanism of melanosome biogenesis in more detail, by investigating the functional relationship between Rab7 and gp100/Pmel17.

In pigmented melanoma cells, wild-type Rab7 and its dominant active mutant (Rab7-Q67 L), but not its dominant negative mutant (Rab7-T22N), were co-localized in the perinuclear region with granules containing the stage I melanosomes where the full-length, immature gp100 /Pmel17 was present. Over-expression of Rab7-Q67L and, to a lesser extent, Rab7-WT increased the amount of proteolytically processed mature gp100. Moreover, siRNA-mediated Rab7 -knockdown considerably inhibited the gp100/ Pmel17 maturation. These results collectively suggest that the GTP-bound form of Rab7 promotes melanogenesis through the regulation of gp100/Pmel17 maturation in melanoma cells [14] (Fig. 1). Two possible mechanisms to promote the gp100/Pmel17-processing can be hypothesized: 1) Rab7 enhances the transport of gp100/ Pmel17 to the stage I melanosomes, and 2) Rab7 promotes the transport of enzymes and their regulators that participate in the processing of gp100/Pmel17. As for the first possibility, gp100 /Pmel17 was reported to have been moved from the TGN to the stage I melanosomes directly and/or indirectly via the plasma membrane and early/late endosomes [7),15].

(3) Biologic activities of tyrosinase, TYRP1 and TYRP2/DCT

It is well known that the synthesis of melanin intermediates through tyrosinase involves the production of cytotoxic free radicals [16),17]. As previously reported [18], when cells are infected with recombinant adenovirus carrying tyrosinase cDNA (Ad-TYR), they deteriorate to the point of death several days after virus inoculation. However, when TYRP1 or TYRP2/ DCT was co-expressed with tyrosinase in melanocytes and melanoma cells, but not in non -melanocytic cells, tyrosinase-mediated cell death was clearly suppressed. Western blot analysis revealed that expression of TYRP1 hardly affected the amount of co-introduced tyrosinase in either the melanocytic or non-melanocytic cells. In cells expressing both tyrosinase and TYRP1 or tyrosinase and TYRP2/ DCT, the amount of total melanin and/or eumelanin increased substantially more than in cells expressing tyrosinase alone. On the other hand, levels of pheomelanin were similar in these three cell types. These findings suggest that TYRP1 and TYRP2/DCT play an essential role in suppressing tyrosinase-mediated cytotoxicity in melanocytic cells, possibly through interaction with tyrosinase in melanosomes, despite the increase of melanin [19].

To further define the functional domain of TYRP1 for tyrosinase-mediated melanin production and cytotoxicity, we constructed recombinant adenoviruses that expressed missense or deletion mutants of human TYRP1. The missense mutants H192L and H377L, which affected N-terminal and C-terminal copper-binding domains respectively, lost the additive activity of TYRP1 to the tyrosinase-mediated melanin production. However, one (H192L) showed suppression against tyrosinase-mediated cyto-

toxicity as effectively as wild-type TYRP1. On the other hand, three missense mutants whose defects were located between the 38th and 517th codons (S38C, C110Y, S354C and D517Y) retained the melanogenic activity, while losing the anti-cytotoxic activity. These results suggest that 1) the functional domains of TYRP1 for the melanogenic activity and the inhibition of tyrosinase-mediated cytotoxicity are different and distinct, and 2) the anti-cytotoxic activity of TYRP1 may not be associated with the specific domain, but with the whole protein structure [20].

2. Induction and suppression of apoptotic cell death of melanoma cells

(1) p53 family members

Melanoma cells rarely contain mutant p53 and hardly undergo apoptosis by wild-type p53. By using recombinant adenoviruses that express p53 or p53-related p51A (p63) or p73β [21], we tested their apoptotic activities in melanoma cells. Yeast functional assay revealed a mutation of p53 at the 258th codon (AAA [K] instead of GAA [E]) in one cell line, 70W, out of six human melanoma cell lines analyzed. Adenovirus-mediated transfer of p53, p63, and/or p73β suppressed growth and induced apoptotic DNA fragmentation of SK-mel-23, SK-mel-118 and 70W cells. Interestingly, p63 induced DNA fragmentation in the cells more significantly than p53 and p73β. Apoptotic Bax and antiapoptotic Bcl-2 were not significantly up- or down-regulated by expression of p53, p63, or p73β, except for p53-expressing 70W cells, which contained a larger amount of Bax protein than LacZ-expressing cells. Activation of caspase-3 was demonstrated only in p63-expressing SK-mel-118 cells. Thus, p63 can mediate apoptosis in both wild-type and mutant p53-expressing melanoma cells more significantly than p53 and p73β. It also suggests that in melanoma cells, cellular target protein(s) other than Bcl-2 and Bax may be responsible for induction of p63-mediated apoptosis, and caspase-3 is not always involved in the apoptosis by p53 family members [22]. We, then, constructed eight different kinds of chimeric p53 and p63 cDNAs to determine which subregion of p63 is essential for p63-mediated apoptosis. Finally, to identify cellular genes that work transcriptionally downstream of p63, we compared RNAs between Ad-LacZ- and Ad-p63-infected SK-MEL-118 cells by differential display and DNA microarray analyses. As a result, more than 20 species of cellular genes were found to be up-regulated more than ten fold in the Ad-p63-infected SK-MEL-118 cells [23].

(2) IFNβ

Although the mechanisms of IFN-mediated cell death have not been fully elucidated, IFN is known as an effective anti-tumor agent. It induces many biological responses by regulating IFN-stimulated genes (ISGs) [24]. Several ISGs, such as double-stranded RNA-activated protein kinase, myxovirus resistance protein A, melanoma differentiation associated gene-5 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) are related to the anti-tumor effects of IFN [25]. It has been reported that IFN-β induces apoptosis in melanoma cells more significantly than IFN-α and IFN-γ do [26]. TRAIL, known as Apo2 ligand, is also a member of the TNF family of transmembrane proteins, and it leads tumor cells to apoptosis by stimulating death receptors [27]. It has been suggested that the involvement of TRAIL is essential for the apoptotic cascade induced by IFNs in certain melanoma cell lines, as well as other tumor cell lines [26]. To characterize the signaling pathway involved in IFN-β-mediated apoptosis, we analyzed the biological effect of IFN-β on the cell death and caspase activation of melanoma cells. Among four human melanoma cell lines tested, MM418, SK-mel-23 and SK-mel-118 showed inhibition of cell viability, increased apoptotic populations and up-regulation of caspase-3 activity by IFN-β. Furthermore, caspase-2 activity was upregulated in all the IFN-β-sensitive cell lines, although MM418, which is IFN-β sensitive, did not show induction of TRAIL mRNA

by IFN-β. On the other hand, G361 was not affected by IFN-β. However, treatment of G361 cells with IFN-β significantly increased their sensitivity against exogenous TRAIL-mediated apoptosis accompanied with activation of caspase-2. Caspase-2 activation is commonly associated with induction of apoptosis in IFN-β-sensitive melanoma cells [28].

(3) NF-κB and TNF-α

The constitutive activation of nuclear factor -κB (NF-κB) is known to be an emerging hallmark of melanoma and plays a pivotal role in many aspects of melanoma tumorigenesis, including protection from apoptosis [29),30)]. A major apoptosis signaling pathway in melanoma cells relies on tumor necrosis factor (TNF)-α and the network associated with TNF receptor (TNFR)-1 signaling [29),30)]. Unlike Fas, which mainly functions as a stimulator of the apoptosis cascade, signaling from TNFR-1 often results in inhibition of apoptosis through the efficient activation of NF-κB. Therefore, the balance between the pro- and anti-apoptotic signals within the TNFR-1 framework is central in dictating whether TNFR-1 activation results in the TNF -α-dependent cell death in melanoma cells.

We investigated the implication of diacylglycerol kinase (DGK) α (type I isoform) in melanoma cells because we found that this DGK isoform was expressed in several human melanoma cell lines but not in melanocytes. Intriguingly, the overexpression of wild-type DGKα, but not its kinase-dead mutant, markedly suppressed TNF-α-induced apoptosis of AKI human melanoma cells. In the reverse experiment, siRNA-mediated knockdown of DGKα significantly enhanced the apoptosis. The overexpression of other type I isoforms (DGKBE-TAβ and DGKγ) had, on the other hand, no detectable effects on the apoptosis. These results indicate that DGKα specifically suppresses the TNF-α-induced apoptosis through its catalytic action. We found that the overexpression of DGKα-WT, but not DGKα-KD, further enhanced the TNF-α-stimulated transcriptional activity of an anti-apoptotic factor, NF-κB. Conversely, DGKα knockdown considerably inhibited the NF-κB activity. Moreover, an NF-κB inhibitor blunted the anti-apoptotic effect of DGKα overexpression. Together, these results strongly suggest that DGKα is a novel positive regulator of NF-κB, which suppresses TNF-α-induced melanoma cell apoptosis [31].

Discussions and perspectives

gp100/Pmel17 serves as the structural foundation of the fibrils in the stage I and II melanosomes and melanogenic enzymes, such as tyrosinase, TYRP1 and TYRP2/DCT. Therefore, they should be transported and enriched in the stage II melanosomes [5]. Patients with Hermansky-Pudlack syndrome type 2 (HPS-2) are reported to carry mutations in the β3A subunit of AP-3 [32]. Since AP-3 coats are detected primarily on early endosomal tubules, and mislocalized tyrosinase in AP-3-deficient cells accumulates in the early endosomes and multivesicular bodies[33),34)], tyrosinase is transported to melanosomes via endosomes not directly to melanosomes from the TGN. It is not known what differentiates between melanosomes and lysosomes in melanocytic cells. It has been reported that gp100/Pmel17 accumulates in the lysosomes of non-melanocytic cells [35].

Although direct physical interactions between Rab7 and melanosomal proteins have not yet been shown, three studies suggest links between them, including the vesicular trafficking of tyrosinase and TYRP1 to melanosomes by Rab7 [10),11)], and the effect of rab7 on the maturation of gp100/Pmel17 [14]. Since Rab7 controls microtubule-mediated transport of the early melanosomes [36], and the maturation of the early and late endosomes [37], it is possible that Rab7 contributes to the effective trafficking of melanosomal proteins and the maturation of gp100/Pmel 17. It is also possible that several factors, which participate in and/or enhance the proteolysis of gp100/Pmel17, are transported by Rab7 to the early melanosomes.

The p53 family members, tumor suppressor

genes, were shown to induce apoptosis in melanoma cells. We have constructed chimeric p53/p63 cDNAs to test if the apoptosis can be induced in melanoma cells more significantly than p63. One of the chimeric p63/53 showed the strongest apoptotic activity in melanoma cells, and it may be possible to identify apoptosis-related genes through microarrays from mRNA in melanoma cells expressing this chimeric molecule. Research concerning this is ongoing.

Stage IV melanoma with dissemination to distant organs is invariably incurable. It is not possible to identify the patients who will respond to the systemic chemotherapy or IFN-β before treatment. In some melanoma cell lines, IFN-β can induce apoptosis in the presence of 5-azacytidine, suggesting that apoptotic genes are transcriptionally suppressed by methylation of genomic DNA [38),39)]. We have been trying to identify the cellular genes that are epigenetically suppressed in melanoma cells, and a novel micro RNA has been characterized (Nishizaka T *et al*, manuscript in preparation). This line of study may contribute to the elucidation of the mechanism of resistance of melanoma against IFN and chemotherapy and to epigenetic therapy.

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