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Amyloids, Melanins and Oxidative Stress in Melanomagenesis

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Abstract

Melanoma has traditionally been viewed as an ultra-violet (UV) radiation induced malignancy. While UV is a common inducing factor, other endogenous stresses such as metal ion accumulation or the melanin pigment itself, may provide alternative pathways to melanoma progression. Eumelanosomes within melanoma often exhibit disrupted membranes and fragmented pigment which may be due to alterations in their amyloid-based striatial matrix. The melanosomal amyloid can itself be toxic, especially in combination with reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by endogenous NADPH oxidase (NOX) and nitric oxide synthase (NOS) enzymes; a toxic mix that may initiate melanomagenesis. Further understanding of the loss of the melanosomal organization, the behavior of the exposed melanin, and the induction of ROS/RNS in melanomas may provide critical insights into this deadly disease.

The color of our skin and hair is largely determined by variations in the two main melanin types, black/brown eumelanin and blond/red pheomelanin [1]. The ratio of these two melanin types is also a major predictor of melanoma susceptibility, with the darker pigmented population significantly less susceptible to skin cancers of all types [2, 3]. But the connection between melanoma and pigmentation is unusual, for instance squamous cell carcinomas and other non-melanoma skin cancers are relatively common in both black and white albinos and yet the development of cutaneous melanoma is rare [4, 5]. These individuals still have melanocytes, but they cannot make melanin; perhaps the carcinogenic progression to melanoma depends on the presence of the pigment itself. Even so-called amelanotic melanomas generate melanin; in cultured human melanoma cells and

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melanocytes pigmentation is observed only when the darker eumelanin is detectable, even when substantial amounts of the lighter pheomelanin are present [6].

Reactive Oxygen Species (ROS) and melanin

The link of melanin generation with melanoma seems at first counter-intuitive, as melanin pigment is in general protective [2, 7, 8]. But the synthesis of melanin has long been recognized as involving cytotoxic molecules and is tightly compartmentalized within pigment-producing cells [9–11]. Both melanocytes and melanoma cells exhibit higher basal levels of ROS as compared to keratinocytes and fibroblasts [12–15]; the source of these ROS, at least in part, results from the melanosome and its contained melanin [16, 17]. Oxidative stress has also been linked to pigmentation disorders, such as vitiligo [18, 19]. Conversely, inhibiting melanin synthesis by N phenylthiourea reduces intracellular ROS in melanocytes [15].

Of the two types of pigment, the black/brown eumelanin plays the major role in protecting skin cells from UV radiation [20]. In contrast, the yellow/red pheomelanin is much less protective; as the pheomelanin to eumelanin ratio increases in isolated melanosomes, the UV absorption capacity decreases [21]. Melanocytes with high pheomelanin content can become pro-oxidant, particularly in the presence of UV radiation [22, 23] and/or metal ions [22, 24]. The fair skin color and and red hair phenotype are associated with non-functional melanocortin 1 receptor gene (Mc1R) [25]; melanocytes from these individuals showed increased ROS generation upon UV radiation [26, 27]. These characteristics of pheomelanin pose a major attributable risk for melanoma for the fair skinned. But still skin cancer, and especially melanoma, are the exception rather than the rule. However, most such individuals, including those with repeated sun-burns and possibly other environmental exposures, never developeg melanoma. Clearly other factors in addition to UV radiation and the pigment itself contribute to melanomagenesis.

Melanosomes and pigment regulation

In melanocytes the pigments are generated within suborganelles called melanosomes through a complex series of tightly regulated processes, controlled by over 120 genes [28, 29]. Ultrastructural investigations have shown distinct differences between eumelanin- and pheomelanin-containing melanosomes. Eumelanosomes are ellipsoidal in shape and display a proteinaceous striatial matrix upon which eumelanin is deposited and ordered in the early stages of development. Pheomelanosomes are typically spherical and the pigment has a coarser granular appearance. Pheomelanosomes also contain significantly more protein than eumelanosomes [30], and the amorphous protein matrix is decidedly more mobile than the fibrillar matrix in eumelanosomes [31]. Melanosomes of both types appear similar before melanization, and contain small vesiculo-globular bodies that appear intimately involved in the melanization process [32, 33]. In early stage eumelanosomes, well-formed fibrils or striations are observed, upon which the black melanin is deposited. This is illustrated in a transmission electron microscopy (TEM) image of a heavily pigmented normal human melanocyte cell line (Figure 1). In melanosomes of mixed phenotypic individuals,

pheomelanosomes are observed with striatial fibrils but exhibited spotty and incomplete melanization [34].

Also shown in Figure 1 are malformed melanosomes isolated from the heavily pigmented melanoma cell line MNT1, with dramatically altered melanosome structures. It has been recognized for some time that melanosomal genesis is altered early in melanoma progression, including abnormal disposition of melanin [35] and a loss of membrane integrity [11, 36–38]. The presence of the melanin precursor cysteinyldopa in the blood has long been recognized as a clinical marker of melanoma progression [39]. There are also rare reports of generalized melanosis as a complication of melanoma, in which melanin precursors are secreted into the tissue of patients, resulting in hyperpigmentation [40]. Melanoma cells of several types demonstrate pro-oxidant behaviors not seen in normal melanocytes or other cancer cell lines [16, 17]. This suggests that an essential change had occurred within transformed melanoma cells that render its melanin more reactive and susceptible to oxidative stress, which we propose is tied to the loss of melanosomal organization (Figure 2). Detailed relationships among the different melanin subtypes and new information on melanosomal proteins is discussed below.

The amyloid-melanin connection

Recent work has shown that the filaments that form the striatial matrix in early stage eumelanosomes are in fact amyloid fibrils [41, 42]. Amyloid is a broad term applied to aggregates of proteins that form extended “cross β” fibrils associated with a number of age-related degenerative diseases such as Parkinson’s and Alzheimer’s diseases [43]. Although the amyloid proteins associated with the various neuro-degenerative diseases are different, the accumulated fibrils identified in each disease share a common parallel β-strand structural motif [44, 45].

The initial evidence for amyloids in melanocytes came from the discovery that the melanosomal-associated protein Pmel is cleaved in normal melanosomes into two fragments, called $\mathbf{M}_{\alpha}$ and $\mathbf{M}_{\beta}$, and that its overexpression in non-pigment cells resulted in the formation of striations within multivesicular bodies[46]. Subsequently the Pmel-derived amyloid fibrils were characterized in normal melanocytes [47]. Mutations of the same proprotein convertase Furin was found in a rare familial amyloidal disease [48], and. Maturation of eumelanosome depends on the cleavage of Pmel [49], and it is fragment $\mathbf{M}_{\alpha}$ which self-assembles to form the striatial fibrils within the early stage eumelanosomes [41]. An important characteristic of the nonpathogenic Pmel-amyloid is that the fibrils form at mildly acidic pH (~5.0) and dissolve at neutral pH, which allows for a reversible aggregation-disaggregation process [50, 51]. Thus loss of melanosomal membrane integrity may affect the stability of the striatial amyloid matrix, as illustrated in Figure 2.

The loss of melanosomal integrity observed in melanoma is particularly intriguing, as similar membranal disruptions are also observed in amyloid diseases, attributed to the action of protofibril precursors which form pores that span the membrane [52–54]. Although pheomelanosomes lack the striatial matrix of the early stage eumelanosomes, both evolve from common precursor premelanosomes and are often found within the same cells [32, 55].

We hypothesize that melanosomal abnormalities seen in melanoma may be due to a loss of or change in amyloid fibril formation. Using antibody stains our initial observations document the presence of amyloid precursors in several melanoma cell lines; these include both heavily pigmented black MNT1 cells [56], and amelanotic SK-Mel28 cells [57]. If substantiated, then amyloid dysfunction may be a characteristic early pathology in melanoma, potentially related to both carcinogenesis and to the high rate of mutation and chemo-resistance of this deadly cancer. These ideas suggest several diverse lines of investigation.

- *Are mutations of proteins regulating the striatial matrix in eumelanosomes involved in melanoma?* Pmel itself was first identified from inbred mice (*silver)* which have accelerated graying of their hair [58]. The expression of a variety of melanosomal proteins has been shown to become altered in malignant melanoma [59, 60]. Mutations in Pmel result in loss of melanin production and damage to the cell membrane, possibly due to changes in the formation of pathological amyloid aggregates [61]. Several mutations of melanin-synthesis related genes are also melanoma-associated, such as in genes for the melanocortin receptor 1 (Mc1R), MiTF, agouti (ASIP) and tyrosinase [62–65]. Mc1R serves as a switch between eumelanin and pheomelanin synthesis, and therefore it plays an essential role in skin color [62, 66–69], although its exact role in melanomagenesis is still under debate [26, 70, 71].
- *Might the aggregation of melanin by deposition on amyloid fibrils inhibit its pro-oxidant and cytotoxic behavior?* If so, then this would explain the varied behaviors of isolated eumelanin and pheomelanins [13, 21, 22, 24, 72, 73]. Melanosomes purified from melanoma generate ROS under ambient conditions, while melanosomes from highly pigmented human melanocytes do not [27]. Disturbed melanin synthesis and chronic oxidative stress are present in dysplastic nevi, a possible first transformative step towards melanoma [72].
- *Is there a connection between ROS/RNS and the loss of melanosomal integrity?* Ultraviolet radiation induces an inflammatory response in skin [74], and melanoma tumors themselves often show macrophage and neutrophil infiltration. Melanocytic cells express NADPH Oxidase (NOX), a key player in the generation of oxidative stress, mainly as NOX1 and NOX4 enzymes and their subunits [75–77]. NOX1 is expressed in all melanoma cell lines examined at a higher level than normal human melanocytes [76], but NOX4 was only detected in a subset of metastatic melanoma samples [76, 77]. NOX1 protein levels increase after UVR in a primary melanoma cell line (Liu-Smith and Meyskens, unpublished data), and may be a major source of UV-induced ROS in dysplastic nevi [78].

  The neuronal form of Nitric Oxide Synthase, nNOS, is found in melanogenic cells and its expression is much higher in melanoma cell lines than in normal melanocytes [79]. Inhibiting nNOS by specific inhibitors led to decreased xenografted melanoma tumor growth *in vivo* [79, 80]. Similarly, NADPH Oxidase (NOX) activity is induced by UV radiation, and NOX1 protein levels are higher in melanoma cells than in normal melanocytes [76, 78]. It is well known that the

toxicity of NO is dramatically enhanced in conjuction with mitochondria-generated ROS [81]. Hence the ROS/RNS pool from various sources may form a detelerious feedback circuit for melanomagenesis, resulting from the leaking of melanosome contents (Figure 2).

Likewise, there is a strong connection between oxidative stress and the formation of amyloid deposits in neurodegenerative diseases [82–85], where the direct binding of Cu(II) ions to the β-amyloid is often implicated [86]. Early studies indicated that β-amyloid deposition caused activation of NOX and release of ROS in a variety of cell lines [87, 88]. Cu ions are abundant in melanosomes because they are required for tyrosinase activity; the combination of amyloid, Cu, and ROS/RNS may form a vicious cycle that serves as a carcinogenic threat to melanocytes [89]. Nevertheless, endogenous ROS can act as preventive agents for melanomagenesis as they kill damaged cells and prevent transformation [90]; in other venue endogenous ROS can be signals for cell proliferation and promote transformation [78, 91]; therefore the function of ROS in melanomagenesis is indeed complicated.

- *Is melanoma an amyloid disease and vice versa?* Melanocytes are derived from the neural crest during differentiation, and thus may be susceptible to causative factors related to neurodegenerative amyloid-based diseases. For instance, it is known that Parkinson's disease patients have a higher risk for melanoma, and vice versa [92, 93]. Therefore we speculate that the neuromelanin in neuronal cells may also exhibit similar redox capacity as eumelanin in the melanocytes, because melanocytes are also of neural crest origin. Emerging evidence implies that neuromelanin is cytoprotective when contained within neuronal cells but causes cytotoxic effect when released by damaged neuron cells [94].

Taken together, the melanomsomal amyloid toxicity may be summarized into several aspects: 1) amyloid changes the structure of the melanosomes and leads to leaking of the melanomsomal contents including amyloid itself, melanin and melanin intermediates; 2) amyloid interaction with ROS and RNS augments the detelerious effect of these species; 3) amyloid may disrupt the cellular membrane leading to altered signal transduction, as has been proposed in other cell types [95, 96].

Conclusions

This viewpoint offers alternative etiological explanations for melanomagenesis, i.e., that amyloidal dysfunction in combination with ROS/RNS generated in situ (via NOX and NOS) may lead to melanomagenesis. Figure 3 charts the relationships among melanin phenotypes and carcinogenic risk, as well as potential links to neurodegenerative diseases. While UV is a common inducing factor via alterations in DNA repair, other stresses such as metal ion or pesticide exposure, may provide alternative stresses that involve other pathways to melanoma progression. The low risk population for melanoma includes dark-skinned individuals who predominantly produce eumelanin, and fair-skinned individuals with predominantly pheomelanin pigment who have low exposure to UV (Figure 3). Fair skinned pheomelanonic individuals are more susceptible to UV-induced stress, especially high dose

blistering childhood sun-burns or intermittent adult life sun-burns that produce moles or freckles. For all populations, unmanaged oxidative stress increases risk for melanoma. The correlation of melanoma and Parkinson's disease may also imply a common causative factor of ROS-amyloid dysfunction in neuronal cells [92]. Further understanding of consequences from the loss of melanosomal organization and the effects of the exposed melanin on melanocytes and neuronal cells may provide critical new insights into both diseases of the melanocytes and neurons.

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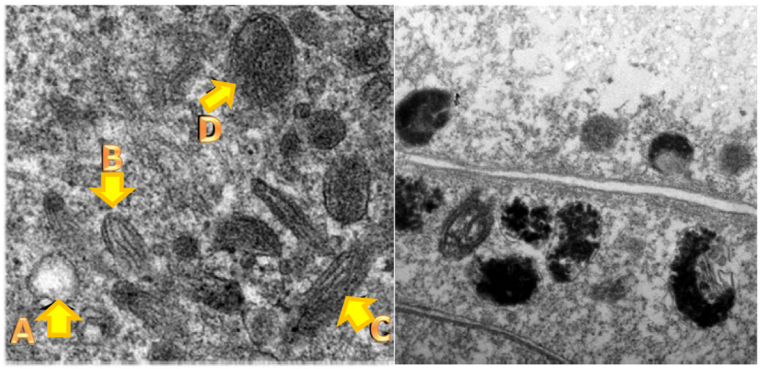

Figure 1. *In situ* transmission electron microscopy images of melanocyte and melanoma cells in culture

Left panel, Stages of normal melanosomes in heavily pigmented melanocyte A: Stage 1, B: Stage 2, C: Stage 3, D: Stage 4. Right panel, abnormal melanosomes in MNT1 melanoma cells: note disruption of structure and difficulty in identifying stages. Adapted from our work Gidanian et al., 2008 [56].

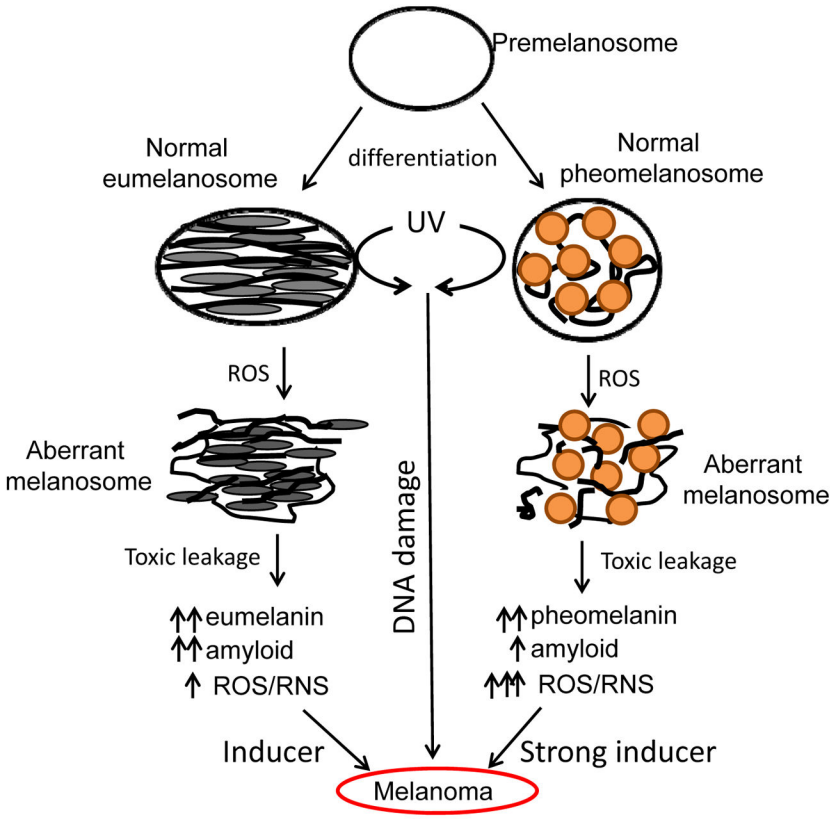

Figure 2. UV/ROS induces aberrant pheomelanosome and eumelanosome structural changes and leads to melanoma transformation/progression

Pre-melanosomes mature and differentiate into eumelanin-contained eumelanosomes and pheomelanin-contained pheomelanosomes, both of which can undergo aberrant structural changes in the presence of UV or ROS, leading to leaking of melanosomal contents (melanins, melanin synthesis intermediates, amyloids, etc) which can further react with ROS and become more toxic. Eumelanosomes may generate more amyloids and less ROS as compared to pheomelanosomes.

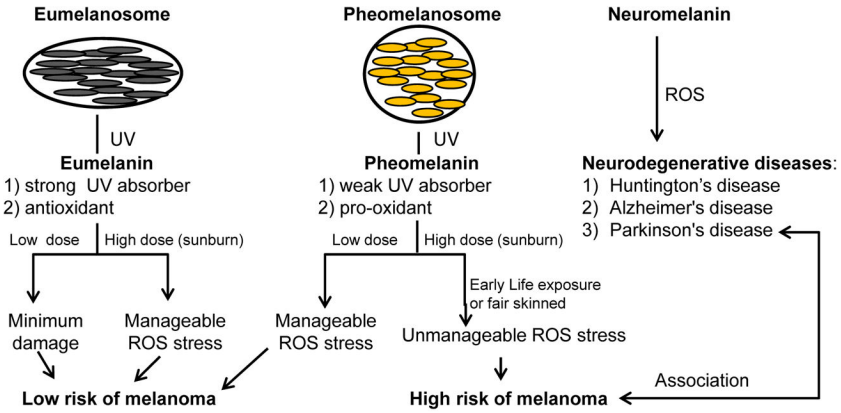

Figure 3. Summary of melanoma risk with melanin types and its potential link with neurodegenerative diseases

Eumelanin strongly absorbs UV radiation and serves as an antioxidant, hence in dark skinned individuals the UV/ROS induced stress is well managed and melanomas are rare. In pheomelanin-predominant light skinned individuals, pheomelanin does not efficiently absorb UV radiation, and upon UV radiation pheomelanin becomes a pro-oxidant. Hence, with high UV doses the stress is difficult to manage and melanoma risk increases greatly in these individuals. A potential link of UV/ROS-induced amyloid toxicity is suggested by the shared risk of some individuals for cutaneous melanoma and Parkinson's disease.