Regulation of Melanogenesis as Studied by Chemical Analysis of Melanins

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INTRODUCTION

Melanin pigments are present in the skin, hair, and eyes and determine their color. It is generally accepted that they function to protect the underlying tissues from the harmful ultraviolet radiation. It is thus important to know how melanins are produced and how this process, melanogenesis, is regulated by genetic, biochemical, and hormonal mechanisms.

Melanins are produced in the melanosome, a specialized organelle, of melanocytes. Melanins are the end products of melanogenesis and therefore characterization of melanins is essential in the study of melanogenesis. I would like to summarize here the usefulness of our chemical method to analyze melanins and its application in studying the regulation of melanogenesis (1, 2). Mammalian melanocytes produce two chemically distinct types of melanin pigments, the black to brown eumelanin and the yellow to reddish pheomelanin. Typical examples are found in hairs of nonagouti black and lethal yellow mice, respectively. However, most of natural melanin pigments are found to be copolymers of these two types of melanin pigments.

Characterization of melanins is rather a difficult problem. Then, why is it so difficult? Among the biopolymers, melanins are unique in many respects. The other biopolymers - proteins, nucleic acids, and carbohydrates - are chemically well characterized; they are composed of distinct monomer units and these units are connected through covalent bonds that can be easily split either by chemical methods or by the action of enzymes. On the other hand, melanin pigments are composed of many different monomer units that are connected through strong carbon-carbon bonds. This makes the systemic characterization of melanins very difficult.

CHEMISTRY OF MELANOGENESIS

Both eumelanin and pheomelanin are derived from the common precursor dopaquinone that is formed by tyrosinase oxidation of tyrosine (Figure 1). Dopaquinone is a highly reactive

intermediate, and in the absence of thiol compounds it undergoes intramolecular cyclization, leading eventually to the production of eumelanin. On the other hand, intervention of cysteine gives rise to the cysteine adducts of dopa, cysteinyldopas. Further oxidation of cysteinyldopas leads to the production of pheomelanin via benzothiazine intermediates.

Eumelanin is a highly heterogeneous polymer consisting of 5,6-dihydroxyindole (DHI) and its carboxylic acid (DHICA) units in a reduced or oxidized state. In addition to tyrosinase, two related proteins, termed tyrosinase-related proteins (TRPs), have been shown to regulate eumelanin formation. Thus, dopachrome tautomerase, or TRP2, catalyzes the tautomerization of dopachrome to DHICA. Oxidative polymerization of DHI is catalyzed by tyrosinase, while oxidation of DHICA appears to be catalyzed by TRP1. It is now clear that the activities of these TRPs greatly affect the quantity and quality of the eumelanin produced. In contrast, no enzymes other than tyrosinase appear to be directly involved in the course of pheomelanin production.

The most critical point in the regulation of melanogenesis is that the addition of cysteine to dopaquinone proceeds much faster than the intramolecular cyclization. These kinetic data indicate that even at a low concentration of 1 micromolar cysteine, this addition reaction proceeds faster. This means that pheomelanogenesis is preferred as long as cysteine is present.

CHARACTERIZATION OF MELANINS

The lack of adequate methods to isolate melanins, their insolubility, and the heterogeneity in their structural features all make the characterization of melanins a rather difficult problem. Comparison of chemical and physical properties of eumelanin and pheomelanin indicates that chemical degradation to produce pyrrole-2,3,5-tricarboxylic acid (PTCA) and aminohydroxyphenylalanine (AHP) appeared most specific. Therefore, in 1985 (3), we developed HPLC methods to quantify these specific degradation products with high sensitivity.

On permanganate oxidation, eumelanin is degraded to form PTCA. The yield is much higher from the DHICA-derived eumelanin than from DHI-derived eumelanin, making PTCA a specific marker of DHICA-derived eumelanin. Oxidation of typical natural eumelanins gives about 2% yield of PTCA, and thus eumelanin content may be obtained by multiplying PTCA content by a factor of 50. On hydriodic acid hydrolysis, pheomelanin is degraded to form AHP. Synthetic pheomelanin gives a 20% yield of AHP, and thus pheomelanin content may be obtained by multiplying AHP content by a factor of 5. Melanin can also be quantified by solubilization in hot Soluene-350 plus water. Absorbance of the solution at 500 nm serves to measure total combined amount of eu- and pheomelanin. Comparison of the HPLC method with the spectrophotometric method shows that in mouse hair, the conversion factors of 45 and 2.5 are more appropriate for PTCA and AHP, respectively and in human hair, they are 160 and 10 (4).

BIOCHEMICAL STUDY OF MIXED MELANOGENESIS

Using these analytical methods, we studied how mixed melanogenesis is controlled at biochemical level. First, we examined the effects of tyrosinase concentration on melanin produced from an equimolar mixture of dopa and cysteine (5). It was shown that when tyrosinase levels were low, cysteinyldopa-genesis was favored. Then, as tyrosinase level became higher, pheomelanogenesis was favored, followed by mixed melanogenesis, as evidenced by the increase of total melanin value. We next studied time course of melanogenesis from an equimolar mixture of tyrosine and cysteine. Tyrosine was gradually decreased while cysteine was consumed much faster because of the oxidation to cystine. After one hour, cysteinyldopa-genesis reached a maximum. Then, cysteinyldopa was oxidized to give pheomelanin at 2 hours. After this period, eumelanin was deposited on the preformed pheomelanin, as evidenced by the increase of total melanin value.

From these results, I wish to propose the hypothesis regarding the effects of tyrosinase activity on the type of melanogenesis. When tyrosinase activity is low, cysteinyldopa-genesis takes place with no or little pigment formation. When tyrosinase activity is a little higher, cysteinyldopa is oxidized to give pheomelanin. And then, when tyrosinase activity is much higher, eumelanogenesis begins to take place. In summary, pheomelanin is always formed first and then eumelanin is deposited on the preformed pheomelanin.

ANALYSIS OF MELANINS AT CELLULAR AND TISSUE LEVELS

This section summarizes some examples of application of our method to the analysis of melanogenesis at cellular and tissue levels. Pigmentation genes are extensively studied in mice; more than 150 mutations in about 60 loci are known in mice (6). As shown in Table 1, this knowledge greatly contributes to understanding the effects of mutations in humans (7). Among genes that are directly involved in pigmentation, agouti and extension genes are most important in the switch between eumelanogenesis and pheomelanogenesis.

α-MSH is a peptide hormone that stimulates melanogenesis to produce the black eumelanin. α-MSH binds to the melanocortin-1 receptor (Mc1r) present on the surface of melanocyte, which activates G protein and then adenylate cyclase, resulting in the elevation of cAMP level and the activation of MITF gene. MITF is known to activate the transcription of tyrosinase and TRP1 genes. Agouti signaling protein antagonizes the action of α-MSH and mahogany protein assists in the effect of agouti signaling protein. Thus, the switch of melanogenesis is regulated by the levels of α-MSH, agouti signaling protein, mahogany protein, and Mc1r activity. This is one example of how biological phenomena are controlled in a complex manner. As shown in the biochemical study, activity of tyrosinase and levels of tyrosine and cysteine are also important in the switch of melanogenesis. On the other hand, activities of TRP1 and TRP2 are important only in eumelanogenesis.

We applied our methods to characterize melanins in hairs from various coat-color mutant mice. The congenic mice have the identical genetic background except for a single gene. Thus, it is an ideal system to study the effects of mutations in pigmentation genes. Black and brown mice produced almost pure eumelanin while lethal yellow and recessive yellow mice produced purest pheomelanin in nature. Chinchilla mutation is known to reduce tyrosinase activity to one-third of the wild type. This mutation of tyrosinase reduced a eumelanin content in black mice to a half while it reduced a pheomelanin content in lethal yellow mice to one-tenth. This is consistent with our hypothesis that at the lowest level of tyrosinase, pheomelanin production is greatly suppressed to produce little pigment. Mahogany mutation suppressed the effect of lethal yellow, resulting in a partial switch to eumelanogenesis. Slaty mutation reduced the levels of total melanin only slightly, while it reduced the levels of PTCA to less than one-fifth of the wild type. This indicates that slaty mutants produce DHICA-poor eumelanin, due to the low dopachrome tautomerase activity.

Next, we analyzed melanin contents in human hair of various colors. Human hairs give a broad spectrum of colors. Black, brown, light brown, and blond hairs differed greatly in eumelanin contents, but they showed only 2-fold difference in pheomelanin contents. Interestingly, suppression of melanogenesis resulted in a partial switch to pheomelanogenesis. Red hair is exceptional in that pheomelanogenesis continues to take place. Recently, it is shown that red hair in human and other higher animals is caused by mutations at MC1R gene, similar to recessive yellow mutation in mice.

We also analyzed melanin contents in cultured human melanocytes taken from dark people and light people. Dark melanocytes produced mostly eumelanic pigments, while light melanocytes produced mixed-type pigments. There were about 10-fold differences in the combined amount of melanins, between dark and light melanocytes. This is consistent with about 10-fold differences in the tyrosinase activity.

CONTROL OF MELANOGENESIS AT THE CELLULAR AND TISSUE LEVELS

Our method has been applied to studying control of melanogenesis at cellular and tissue levels. The following examples are the results of collaboration with several laboratories (2).

In 1986 (8), Burchill, Thody, and I examined the effects of α-MSH on melanogenesis in viable yellow mice. This mutation at agouti locus is unique in that neonatal and adult mice produce yellow hair while pubertal mice produce brown hair. When pubertal mice were injected with α-MSH, tyrosinase activity was increased 1.7-fold with a production of more eumelanic hair. When these pubertal mice were injected with bromocriptine that reduces α-MSH secretion, tyrosinase activity was reduced to 8% of the control with a production of more pheomelanic hair. Similarly, neonatal mice also responded to the MSH treatment. It is interesting to note that the amount of melanin produced correlates well with the tyrosinas activity. These results indicated, for the first time, the significance of tyrosinase activity in the control of melanogenesis.

In 1991, Thody's group analyzed melanin in human epidermis and showed the presence of pheomelanin in the skin for the first time (9). They found that the relative ratios of eumelanin to pheomelanin in epidermis correlated well with those found in hair taken from the same subjects. They also examined the effects of PUVA therapy on melanogenesis in human epidermis. Eumelanin content was increased 2.6-fold while pheomelanin content was increased only 1.6-fold, which showed that the PUVA therapy promoted eumelanogenesis.

Hunt and Thody also examined the effects of superpotent synthetic α–MSH analogue on melanogenesis in human melanocytes. Treatment of cells with the synthetic MSH resulted in an increase of eumelanin content in all of these 6 cell lines from different ethic origins. On the other hand, pheomelanin showed a variable response. This resulted in a clear, significant shift to more eumelanic cells.

Thody's group also examined the effects of agouti signaling protein on melanin contents in B16 melanoma cells. Treatment of cells with α-MSH resulted in the increase of melanins, especially of eumelanin. However, this increase was almost completely suppressed by the addition of a high level of agouti signaling protein. Agouti signaling protein also inhibited melanogenesis in the absence of α-MSH. These results indicate that agouti signaling protein can bind directly to melanocortin-1 receptor.

Next, we examined the effect of tyrosine concentration on melanogenesis. When grown in a medium containing a 0.01 millimolar tyrosine, LND1 cells were colorless. But the cells were actually pheomelanic as shown by the high AHP value. When tyrosine concentration was increased to 0.2 millimolar, pheomelanin content was increased by 5- to 10-fold and cells became highly pheomelanic and looked yellow. When tyrosine concentration was increased further to 2 millimolar, only eumelanin content was increased by 5- to 10-fold and cells became mixed-type and looked dark brown. These results are consistent with the results of biochemical study, in that both tyrosine and cysteine are initially consumed to form pheomelanin and only after depletion of cysteine, excess tyrosine is oxidized and deposited on the preformed pheomelanin.

Del Marmol, Ghanem, and we examined the effects of cysteine deprivation to one-tenth and one-hundredth of the normal level of 206 micromolar. With such decrease of cysteine concentration, intracellular cysteine and GSH concentrations were decreased to almost zero. This decrease of thiol compounds resulted in a gradual shift to more eumelanic cells, as shown by the decrease of pheomelanin and increase of eumelanin. These results can be interpreted in the reciprocal way, thus, that supplementation of cysteine favors pheomelanogenesis.

We also analyzed the melanin contents in epidermis from 13 human subjects and the corresponding melanocyte cultures. Interestingly, the AHP to PTCA ratios in cultured melanocytes were approximately 10 times higher than those in the corresponding epidermis in

vivo. This indicates that culturing human melanocytes results in a shift to more pheomelanic cells. This may be ascribed to the high cysteine to tyrosine ratio in the culture medium.

To interpret these results, we wish to propose the hypothesis summarized in Figure 2. When the tyrosine level is high or the cysteine level is low, melanogenesis passes rapidly through the initial pheomelangenesis phase, which results in more eumelanic cells. On the other hand, when the tyrosine level is low or the cysteine level is high, pheomelanogenesis takes place as long as cysteine is present. As tyrosinase activity becomes lower, melanogenesis is suppressed to the phase in which only cysteinyldopa production is evident and little or no pigment is produced.

SUMMARY

- Biochemical studies show that in the process of mixed melanogenesis, cysteinyldopas are produced first which are next oxidized to give pheomelanin. After all of the cysteine is consumed, eumelanin is then deposited on the preformed pheomelanin.
- In vitro and in vivo studies show that tyrosinase activity is the most important factor that regulates the switch of melanogenesis, with higher activities increasing melanogenesis, especially eumelanogenesis.
- In culturted melanocytes, the tyrosine to cysteine ratio is critical in determining the eumelanin to pheomelanin ratio.
- Our HPLC method to analyze eumelanin and pheomelanin has become a useful tool in the study of melanogenesis regulation.

There are many problems to be solved before we fully understand the regulation of melanogenesis. Mutations in mouse models are ideal models for studying the genetic and molecular control of melanogenesis. Even in the mouse models, it is not known how cysteine is excluded from being incorporated into melanins in black and other eumelaninc mice. Conversely, it is not known how cysteine is continuously incorporated into pheomelanin in lethal yellow and recessive yellow mice.

ACKNOWLEDGEMENTS

This review is mostly based on my review that will appear soon in the journal Pigment Cell Research (2). The results presented here have been obtained by collaborative studies with many pigment cell scientists. Special thanks are due to Drs. Kazumasa Wakamatsu and Hiroyuki Ozeki of Fujita Health University.

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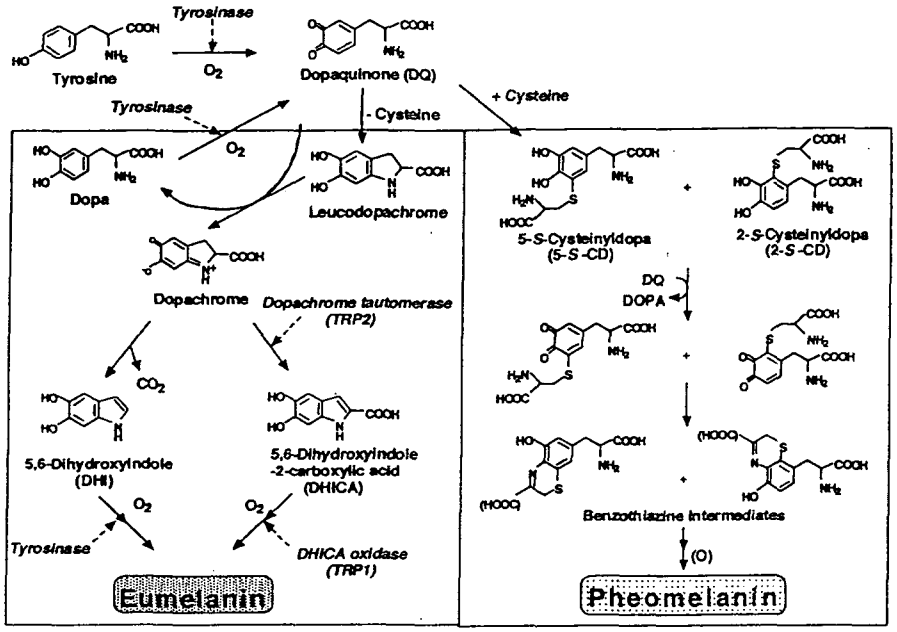

Figure 1. The biosynthetic pathways to eumelanin and pheomelanin. Note that activities of tyrosinase, TRP2, and TRP1 are involved in the production of eumelanin, while only tyrosinase activity (and presence of cysteine) is necessary for the production of pheomelanin.

Table 1. Pigmentation genes in mice and mutations in humans

Mouse gene	Gene product	Human mutation. (Pigmentation)
albino/Tyr	Tyrosinase	Oculocutaneous albinism 1 (OCA1)
slaty/Dct	Tyrosinase-related protein-2 (TRP2)	None described
brown/Tyrp1	Tyrosinase-related protein-1 (TRP1)	OCA3
Agouti (A)	Agouti signaling protein (ASP) (Mc1r antagonist)	None described
extension/Mc1r	Melanocortin-1 receptor (Mc1r)	Skin type I, Red hair
mahogany/Atrn	Mahogany protein (Attractin)	None described
pink-eyed dilution (p)	Melanosomal membrane protein (Transport and stabilization of tyrosinase?)	OCA2
silver (si)	Melanosomal protein	None described

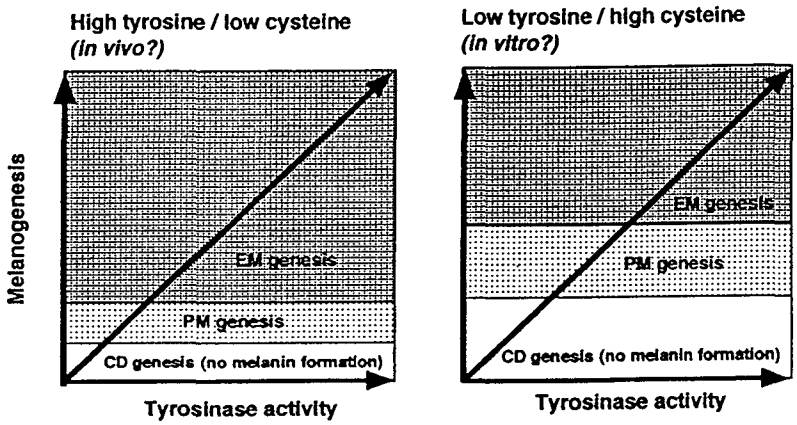

Figure. 2. Hypothesis regarding the effects of tyrosinase activity and concentrations of tyrosine and cysteine on the type of melanogenesis.