Non-Melanosomal Regulatory Factors in Melanogenesis

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The enzyme activity of tyrosinase, catalase, and peroxidase and the number of metal ions in melanogenic subcellular compartments in pigment cells were measured. Coated vesicles were richest in tyrosinase and catalase, whereas pre-melanosomes had the highest amount of peroxidase. Among metals ions examined, copper, zinc, and iron were more concentrated in pre-melanosomes than in coated vesicles. A quantitative analysis revealed that peroxidase served to enhance eumelanin polymer formation from monomers in the presence of hydrogen peroxide and metal ions, especially copper ions, which had the greatest enhancing effect on the conversion of monomers to polymers. *J Invest Dermatol 100:274S–280S, 1993*

Despite the presence of tyrosinase within various sub-cellular compartments in pigment cells, melanin polymer formation can only be detected within pre-melanosomes (PMS). We previously reported that eumelanin monomers and tyrosinase were richer in coated vesicles (CV) than in PMS and melanosomes (MS) [1,2]. From these findings we can hypothesize with regard to the melanin polymer – forming process that there are certain inhibiting factors in CV and possibly some enhancing factors in PMS. On the basis of *in vitro* chemical reaction studies, Prota reported the enhancing function of metal ions for the rearrangement of dopachrome, which is the reactive product of dihydroxyphenylalanine (dopa) [3]. Moreover, peroxidase can enhance the formation of eumelanin polymers from monomers such as 5,6-dihydroxyindole (DI) and 5,6-dihydroxyindole-2-carboxylic acid (DICA), and this polymer-forming process requires hydrogen peroxide [4]. Further, it was proposed that catalase can inhibit eumelanin polymer formation by destroying hydrogen peroxide, which is possibly the substrate most rich in oxygen for the auto-oxidization process in eumelanin polymer formation (G. Prota, personal communication, 1990); however, the actual role of these regulatory factors has not been clarified within pigment cells *in vivo*.

In this review, we report the intracellular site of activity of these regulatory factors in the various melanogenic compartments, such as CV, PMS, and MS, in relation to the polymer-forming process.

MATERIALS AND METHODS

Melanoma Tissues We implanted 20–30 mg of Greene's melanoma into the subcutaneous tissue (both shoulder and flank) of Syrian golden hamsters. The tumors were allowed to grow until they reached a size of approximately $30 \times 20 \times 15$ mm. For histochemical use, we prepared human metastatic melanotic and amelanotic melanoma tissues.

Histochemical Staining of Melanoma Tissues

Catalase: Light microscopic studies were performed using indirect immuno-histochemistry with primary rabbit immunoglobulin G antibody directed against human erythrocyte catalase. Frozen melanoma tissues were cut at 20 μm thickness, mounted on 0.01% poly-L-lysine-coated slide glasses, dried at room temperature, and then washed in 0.1 M phosphate buffer three times each for 5 min at 4 °C. The samples were placed in 100% acetone for 3 min at 4 °C and washed in the same manner, followed by the addition of 7.5% hydrogen peroxide containing 0.1 M phosphate buffer, and then were washed again. The tissues were incubated overnight at 4 °C with anti-catalase antibody diluted twice in 0.1 M phosphate buffer. After the fourth washing, the tissue was placed for 2 h at 4 °C in peroxidase-conjugated Affinipure $F(ab')_2$ fragment anti-rabbit immunoglobulin G (heavy and light chain) that was immunized in goats and diluted 100 times. Following the fifth washing, a Vecta-stain glucose oxidase kit (iodonitrotetrazolium violet) was used for 20 min at 4 °C for color staining. For optimal electron microscopic observation of the distribution of catalase, we chose the pre-embedding method. Melanoma tissues were treated by same method as described above, and 0.05% saponin was added during each step of the treatment process to permit high-molecular-weight antibodies to pass through the cell membrane. As a color substrate we chose diaminobenzidine containing 0.05 M Tris (hydroxymethyl) aminomethane (Tris)-HCl buffer (pH 7.6) for 5 min at 4 °C. Then the melanoma tissues were routinely embedded in epon. All specimens were examined using the JEOL 100S model electron microscope.

Peroxidase: We chose Fahimi's method for histochemical staining to detect internal peroxidase activity [5]. The reaction mixture consisted of 5 mg of 3.3′-diaminobenzidine)-4 HCl, 7 μl of 30% hydrogen peroxide solution, and 10 ml of 0.1 M Tris buffer (pH 8.5). The reaction time was 3 h at a temperature of 37 °C. For preparation of electron microscopic observation, we carried out a routine epon embedding procedure after these treatments.

Preparation of Subcellular Fractions Melanoma tissue was homogenized in an ice-cold solution of MES buffer containing 0.1 M MES, 1 mM ethyleneglycol-bis-(-amino-ethylether)N,N′-tetra-acetic acid, and 0.5 mM $MgCl_2$ (pH 6.8). CV were isolated by the method of Usami *et al* [6]. Purification was carried out at 4 °C. Tumors were homogenized in 1 vol of MES buffer with an additional 0.02% sodium azide. The homogenate was then centrifuged at $20\,000 \times g$ for 30 min, and the resulting supernatant was centrifuged at $100\,000 \times g$ for 60 min. The pellet was suspended in 10 ml of MES buffer containing 10% sucrose. The C V fraction was precipitated by centrifugation for 60 min at $100\,000 \times g$. The pellet was then homogenized in a small volume of the buffer and layered on top of a continuous 30–50% sucrose density gradient (in the MES buffer). The gradient was centrifuged for 16 h at $50\,000 \times g$. A turbid band at about 35–40% sucrose was collected, diluted four times with the buffer, and concentrated by centrifugation at $100\,000 \times g$ for 60 min. The pellet was resuspended, layered on top of a continuous 5–30% sucrose density gradient, and centrifuged at $100\,000 \times g$ for

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Abbreviations: CV, coated vesicle(s); DI, 5,6-dihydroxyindole; DICA, 5,6-dihydroxyindole-2-carboxylic acid; dopa, dihydroxyphenylalanine; GERL, Golgi-associated endoplasmic reticulum lysosome; MES, 2-(N-morpholino)ethanesulfonic acid; MS, melanosome(s); PMS, premelanosome(s); r-ER, rough endoplasmic reticulum; Tris, Tris (hydroxymethyl) aminomethane

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60 min. A band at the density of approximately 15–20% sucrose was collected, pooled, and precipitated by centrifugation. The purified CV were resuspended in a MG^{++}-free MES buffer. PMS and MS were isolated by sucrose density gradient ultra-centrifugation, and the purity of each fraction was examined by electron microscopy, as described previously [7]. Protein was measured by Lowry's method [8].

Metal Analysis Analysis of the metals in the melanogenic compartments of pigment cells was performed using a highly sensitive assay system, namely, inductively coupled plasma mass spectrometry. We used a SPQ6500 mass spectrometer from Seiko Instruments (Kameido, Tokyo, Japan), an instrument by which metals can be measured at a detection limit of 1 ppb (w/w). We added 0.1-ml aliquots of the fractions containing CV, PMS, or MS to 0.3 ml of $HC1O_4$ and 0.6 ml of H_2O_2, and incubated them at 75 °C for 1 h. Then, 4 ml of nanopure water was added, and the solution was filtered through a 0.22 μm Millipore filter. To avoid contamination, we used newly prepared reagents, and all tubes were rinsed in 1 N HCl and nanopure water.

Melanin Polymer Formation Melanin polymer formation from melanin monomers DI and DICA were measured with various metal ions added to the solution. The reaction mixture consisted of 10 mM phosphate buffer (pH 6.8) containing 3.3 μg DI or DICA/ml. In some experiments, hydrogen peroxide was added to the final concentration of 10 mM. The melanin polymer formation was assayed at 37 °C by measurement of the melanochrome absorbance at 540 nm. For the spectrophotometry, we used a Hitachi U-3210 spectrophotometer. At the same time, we carried out the same experiments using various concentrations of horseradish peroxidase.

Electron Microscopy Each subcellular fraction pre-fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and stained by 1% pararungstic acid dissolved in 0.1 M phosphate buffer (pH 7.4). After mounting the samples on microslides, electron microscopy was performed with a JEOL 100S model.

Quantitative Analysis of Catalase We prepared each melanogenic subcellular fraction from hamster Greene's melanotic melanoma. Catalase activity was measured spectrophotometrically using a Hitachi U-3210. The measured peak absorbance was 240 nm, which is the peak of hydrogen peroxide. We observed a decrease in hydrogen peroxide catalyzed by catalase in each fraction containing solution for the initial 30 s at 25 °C. The reaction mixture contained 10 mM hydrogen peroxide, 0.1 M phosphate buffer (pH 6.8), and fraction. Catalase activity was determined by the velocity of the decrease of hydrogen peroxide for the initial 30 s, excluding pseudocatalase activity such as that caused by metal ions. The unit of catalase activity was indicated as minus millioptical density (mOD)/initial 30 s/mg protein in each reaction mixture. Protein was measured by Lowry's method [8].

Quantitative Analysis of Peroxidase We prepared each subcellular melanogenic compartment fraction from hamster melanotic melanoma. Peroxidase activity was measured spectrophotometrically using a Hitachi U-3210, according to the guaiacol test (Devlin's method) [9]. The peak absorbance was 470 nm, which is the peak of the reaction products after this test. The reaction mixture consisted of 20 mM guaiacol, 10 or 40 mM hydrogen peroxide, 10 mM phosphate buffer (pH 6.8), and each fraction containing solution. The reaction monitoring time was 2 min at 25 °C. Protein was measured by Lowry's method [8].

Tyrosinase Assay Measurement of tyrosinase activity for each fraction was carried out following Prota's method (G. Prota, personal communication, 1990). The activity was monitored spectrophotometrically for 10 min at 37 °C at absorbance of 475 nm, which is the peak absorbance of dopachrome. The reaction mixture consisted of 1 mM tyrosine, 3 mM dopa as a cofactor, 0.1 M phosphate buffer (pH 6.8), and one of three subcellular fractions (CV, PMS, or MS).

RESULTS

Catalase The red reaction products in cytoplasm of human metastatic amelanotic melanoma cells treated by polyclonal catalase antibody can be clearly observed under the light microscope (Fig 1). Further examination of catalase activity using immunoelectron microscopic analysis reveals moderately electrondense reaction products appearing on ribosomes of the rough endoplasmic reticulum (r-ER) and on the membrane of some CV, whereas PMS and MS exhibit a negative reactivity (Fig 2). In the case of human metastatic melanotic melanoma cells, the same positive results were seen but were slightly weaker. A histochemical analysis of the localization of catalase in various types of melanoma cells is summarized in Table I. We found that amelanotic cells were strongly catalase positive at the r-ER and in some CV, whereas melanotic cells contained overall less catalase than amelanotic cells. PMS and MS were almost totally catalase negative in both amelanotic and melanotic cell lines examined.

Fractions of CV (Fig 3), PMS, and MS were prepared and analyzed under the electron microscope to spectrophotometrically measure tyrosinase and catalase activity. The result of a quantitative analysis of tyrosinase activity indicated in μM dopachrome converting ability/minute/mg protein for each fraction is summarized in Fig 4. Of all fractions examined, the CV fraction exhibited the highest tyrosinase activity. Similarly, a comparison of the catalase activity of each fraction reveals that the C V fraction had approximately four or five times higher catalase activity than the PMS and MS fractions (Fig 5). These results further support the results of our histochemical analysis of the distribution and localization of catalase in melanogenic subcellular compartments in pigment cells.

Peroxidase Histochemical analysis of peroxidase distribution in melanogenic subcellular compartments of amelanotic melanoma cells, which showed almost no peroxidase activity, and of melanotic melanoma cells, which were found to be rich in peroxidase, reveals the strongest peroxidase activity in PMS for both cell lines. These results are summarized in Table II. An electron microscopic photograph of human metastatic melanotic melanoma cell shows the reaction product on PMS or MS (which cannot be distinguished from each other owing to their dense reaction products and melanin), and on some membranes of the CV (Fig 6a). An enlarged electron microscopic photograph of the same cell is shown in Fig 6b. The subsequent tyrosinase assay of each fraction expectedly shows the highest tyrosinase activity in the CV fraction (Fig 7). Conversely, the peroxidase activity of the CV fraction determined by the guiacol test was much lower than the PMS fraction. The CV fraction exhibited approximately 1.5 times lower

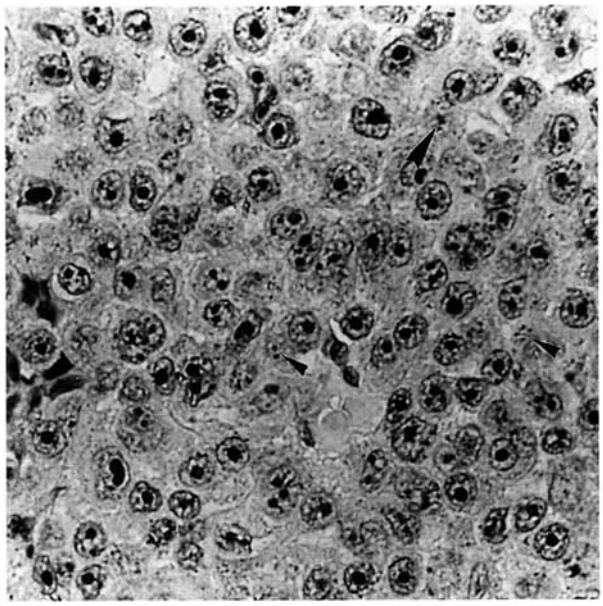


Figure 1. Light microscopic photograph of human metastaric amelanotic melanoma cells treated by polyclonal catalase antibody. Fine granular reaction products *(arrowheads)* can be seen in the cytoplasm. (Magnification × 400.)

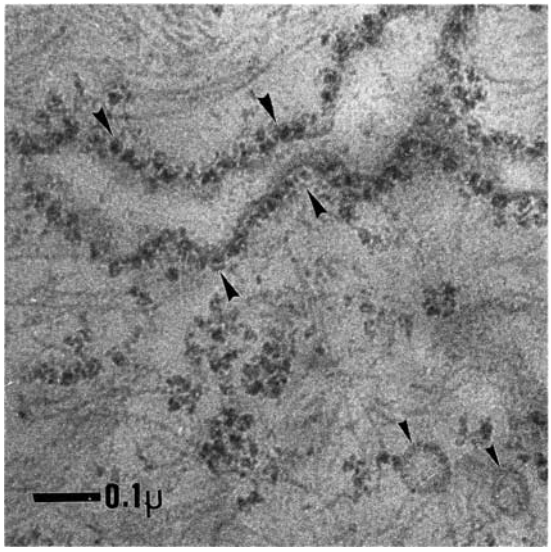


Figure 2. Electron microscopic photograph showing a human metastatic amelanotic melanoma cell treated by immunoelectron microscopic method using polyclonal catalase antibody. Fine granular reaction product *(large arrowheads)* is seen on the ribosomes of the r-ER. The reaction product *(small arrowheads)* can also be seen on the membrane of the CV.

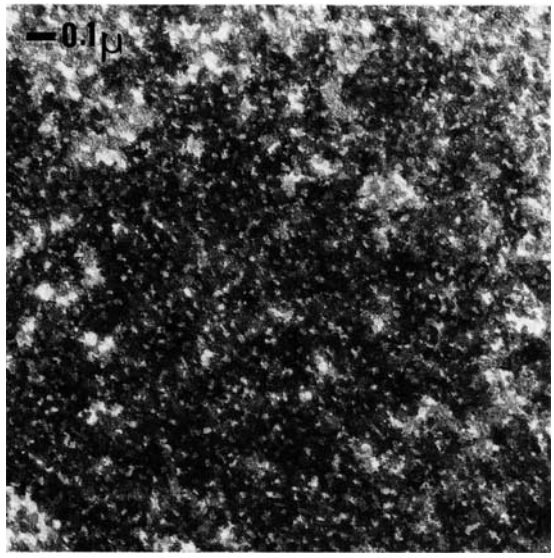


Figure 3. Electron microscopic photograph of CV fraction from hamster Greene's melanoric melanoma. Fraction was fixed by 2% glutarlaldehyde-0.1 M phosphate buffer and stained by 1% paratungustic acid. Many small, low-electron –dense vesicles approximately 40 and 80 nm in diameter are CVs.

Table I. Histochemical Analysis of Localization of Catalase in Various Melanoma Cells

Material	r-ER	CV	PMS	MS
Human metastatic amelanotic melanoma	+++	++	–	–
Hamster amelanotic melanoma	+++	++	–	–
Human metastatic melanotic melanoma (2 cases)	+	+	–	–
Hamster melanotic melanoma	+	+	–	–

peroxidase activity than stage II (faintly melanized) or stage III (moderately melanized) PMS, whereas the MS fraction indicated almost no activity (Fig 8). Further analysis shows eumelanin polymer formation from DI and DICA with and without the addition of hydrogen peroxide (Fig 9a, b). With hydrogen peroxide, peroxidase had no enhancing effect on eumelanin polymer formation; however, with the addition of 10 mM hydrogen peroxide, peroxidase showed a remarkable enhancing effect on eumelanin polymer formation. When either DI or DICA as in the presence of 10 mM hydrogen peroxide without peroxidase, no polymer-enhancing effect was observed.

Metal Ions A comparative detection of copper, iron, and zinc in melanogenic compartments reveals that in relation to CV, MS contained approximately four to six times more metal ions, whereas PMS contained approximately 20–30 times more metals (Fig 10). Among the three metals, cooper had the lowest concentration in all subcellular compartments. The melanochrome formation from DI and DICA after the addition of Fe^{++}, Fe^{+++}, Zn^{++}, and Cu^{++} salts (Fig 11) further shows Cu^{++} to be the most detectable metal in melanin polymer-formation activity, followed by Zn^{++}. Melanin formation from DI and DICA after treatment with various concentrations of Cu^{++} is summarized in Fig 12. When 0.16 mM copper sulfate was added to the indole solutions, DI showed a five-times higher absorbance at 540 nm compared with DICA. Because polymer formation is an oxidative process, we added hydrogen peroxide to Cu^{++} and observed that an increase in melanin formation occurred. When adding hydrogen peroxide to Fe^{++} or Zn^{++}, no obvious enhancing effect on melanin polymer formation was seen.

DISCUSSION

Since the discovery of the melanosome (1961) as the specific organelle in which Seiji, Fitzpatrick, and co-workers believed melanin biosynthesis to occur [10], melanin research has rapidly expanded due to many issues left unanswered regarding the regulatory factors involved in the process of melanogenesis. We recently showed evidence for regulatory factors that exist in non-melanosomal melanogenic compartments, such as the Golgi-associated endoplasmic reticulum lysosome (GERL) and CV system.

In the early stages of melanin research, the only key enzyme was thought to be tyrosinase [11], which activated the process of melanin synthesis in the melanosomes. Using a totally new approach to investigate this hypothesis, Mishima observed the three-dimensional structure of the pigment cell under high-voltage electron microscopy using a goniometer. A number of tyrosinase glycosylation inhibitor experiments viewed under the electron microscope revealed that melanogenic subcellular compartment actually consists of two systems. One system is responsible for the production and glycosylation of tyrosinase; the other is the premelanosome-forming system [12–14].

Tyrosinase was found to be formed in the ribosomes at the r-ER and activated in the GERL. After glycosylation processing, tyrosinase is transferred by pinched-off membraneous compartments known as CV. PMS, on the other hand, are formed by budding off from the Golgi apparatus or smooth endoplasmic reticulum and fusing with CV, which are rich in active tyrosinase, thereby forming melanin polymer [12–14].

Recently, it was suggested that catalase can inhibit melanin polymer formation, which is a series of oxidation processes, because catalase is a universally existing enzyme, mainly responsible for the rapid destruction of cytotoxic hydrogen peroxide (G. Prota, personal communication, 1990). This hypothesis and our own *in vitro* experiments lead us to infer that if the oxygen donor in the eumelanin polymer-formation process is hydrogen peroxide, and catalase is rich in the membrane of CV, then the catalase destroys hydrogen peroxide, thereby preventing eumelanin polymer from forming within the eumelanin-monomer-rich CV. At the

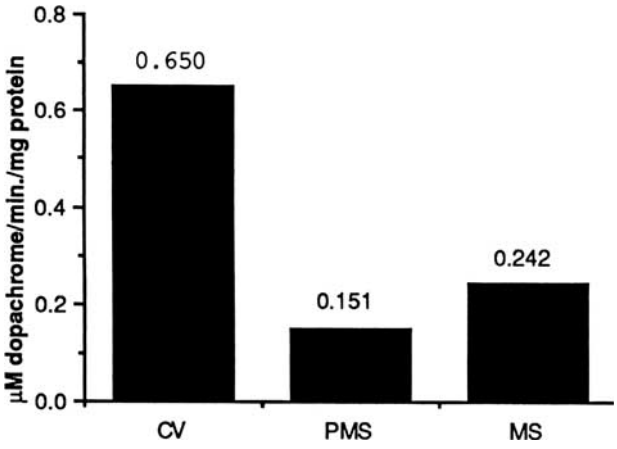


Figure 4. Result of quantitative analysis of tyrosinase activity in each melanogenic subcellular compartment: CV, coated vesicles; PMS, premelanosomes; and MS, melanosomes.

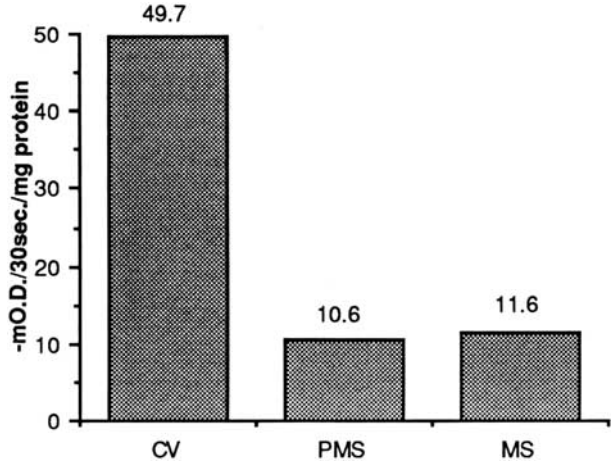
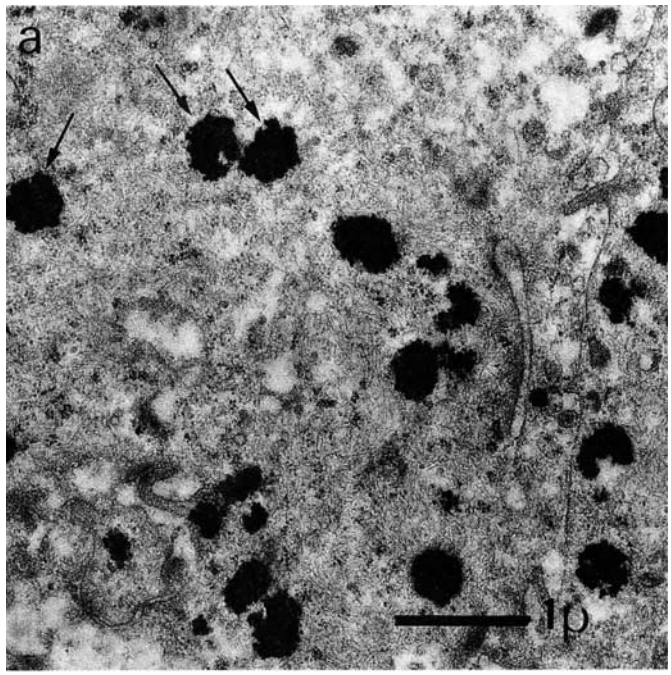


Figure 5. Catalase activity in each melanogenic subcellular compartment (abbreviations as in Fig 4).

Table II. Histochemical Analysis of Peroxidase Distribution in Melanogenic Subcellular Compartments of Amelanotic and Melanotic Melanoma Cells

Material	CV	PMS	MS
Human metastatic melanotic melanoma (two cases)	+	++	+
Human metastatic amelanotic melanoma	–	–	–



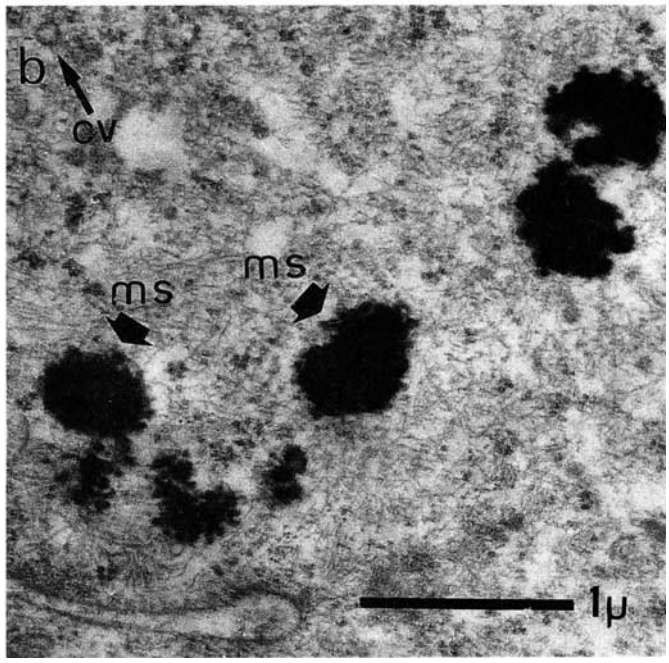


Figure 6. Electron microscopic photograph of a human metastatic melanotic melanoma cell treated by Fahimi's method to detect internal peroxidase activity, *(a)* Near melanosomes, electron-dense reaction product can be seen *(arrows), (b)* Enlarged electron microscopic photograph of the same cell shows reaction product on PMS *(large arrows)* and at the membrane of some CV *(small arrow)*.

same time, however, catalase protects eumelanin monomers in CV from hydrogen peroxide.

We have found other indications of the possible suppressive effects that catalase may have on melanogenesis, such as the high catalase activity in amelanotic melanoma cell compared with melanotic melanoma cells. Moreover, of all subcellular compartments, CV showed the strongest catalase activity.

It may be premature to conclude that catalase plays an inhibitory role in melanin-polymer formation. Our results do indicate, however, that despite the possible presence of enhancing factors, such as peroxidase and metal ions, for eumelanin-polymer formation, catalase is highly localized within the CV and has the capability to inhibit the oxygen supply from hydrogen peroxide, which in turn inhibits a series of autooxidation and/or tyrosinase activity, thereby preventing the formation of eumelanin polymers from monomers. We presume that eumelanin monomers are freed from high catalase activity for the first time in the

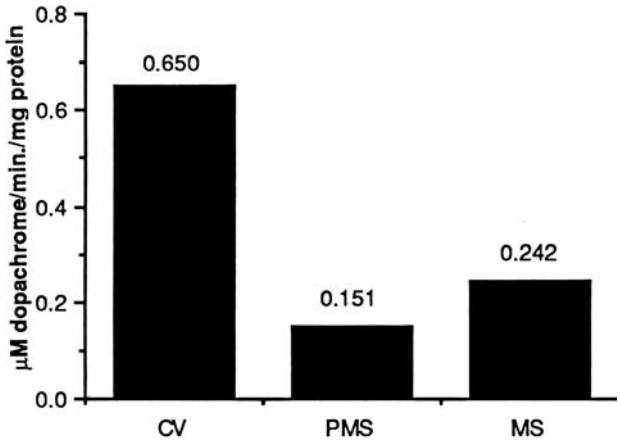


Figure 7. Result of tyrosinase assay of each melanogenic subcellular compartment. CV fraction had the highest tyrosinase activity among all fractions (abbreviations as in Fig 4).

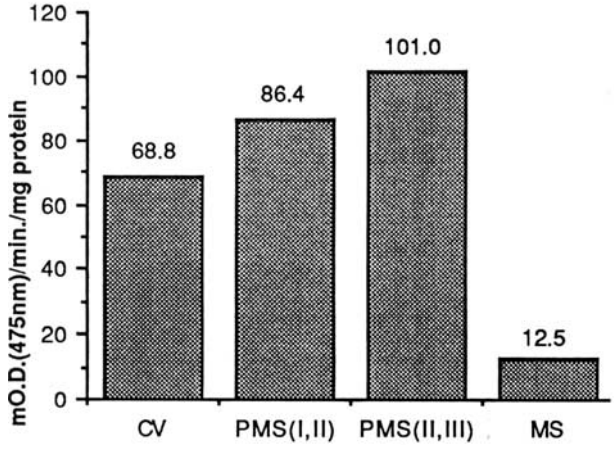


Figure 8. Result of the guaiacol test for peroxidase activity. CV fraction was 68.8 m O.D./minute/mg protein and, depending on stage of development *(roman numerals),* the PMS fraction showed higher peroxidase activity than the CV fraction, whereas the MS fraction showed almost no activity (abbreviations as in Fig 4).

PMS. It is reasonable to infer that after transfer to the PMS, eumelanin monomers are occupied with adequate oxygen from hydrogen peroxide, which is produced perhaps by peroxidase and/or by metal ions, thereby contributing to eumelanin-polymer formation.

The role of peroxidase as an enhancing factor of melanogenesis has been investigated for many years. Ito discovered peroxidase activity in areas of melanin formation [15], and Okun proposed that peroxidase can mediate the formation of melanin from either tyrosine and dopa [16]. Although these proposals were neglected for many years, it was demonstrated that tyrosinase and catalase B(gp 75) are localized in MS [17]. Moreover, Halaban *et al* found that b-locus codes for glycoprotein with the activity of catalase and catalase B are identical to gp75, a known human melanosomal glycoprotein [18]. According to our results, the highest degree of peroxidase activity was found to be in stage II or III PMS. Amelanotic melanoma cells, on the other hand, showed no remarkable peroxidase activity histochemically compared with melanotic cells. Further, *in vitro* biochemical assay of eumelanin-polymer

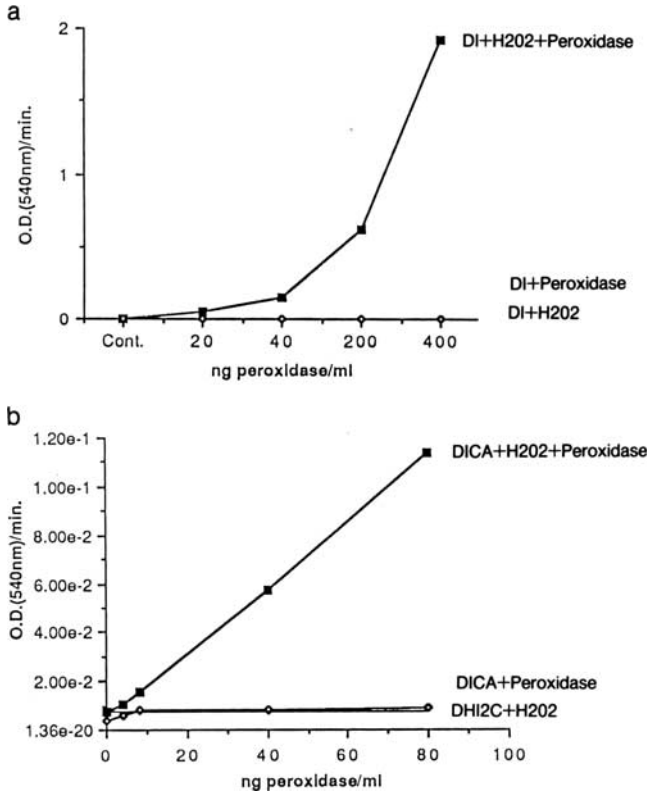


Figure 9. Result of eumelanin polymer formation assay from 5,6-dihydroxyindole (DI) *(a)* and 5,6-dihydroxyindole-2-carboxylic acid (DICA) *(b).* Peroxidase is only able to enhance the effect of eumelanin polymer formation in the presence of hydrogen peroxide (H2O2). Cont., control; O.D., optical density.

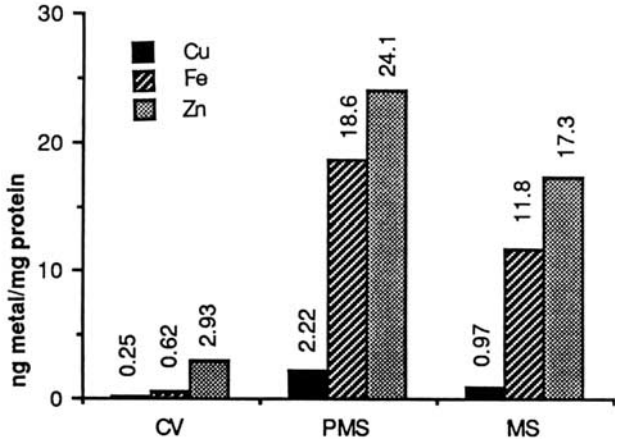


Figure 10. Result of quantitative analysis of copper, iron, and zinc in melanogenic subcellular compartments. The CV fraction contained the lowest amount of these metals among all non-melanosmal melanogenic compartments (abbreviations as in Fig 4).

formation demonstrated that peroxidase can enhance eumelanin-polymer formation from DI or DICA with the coexistence of hydrogen peroxide.

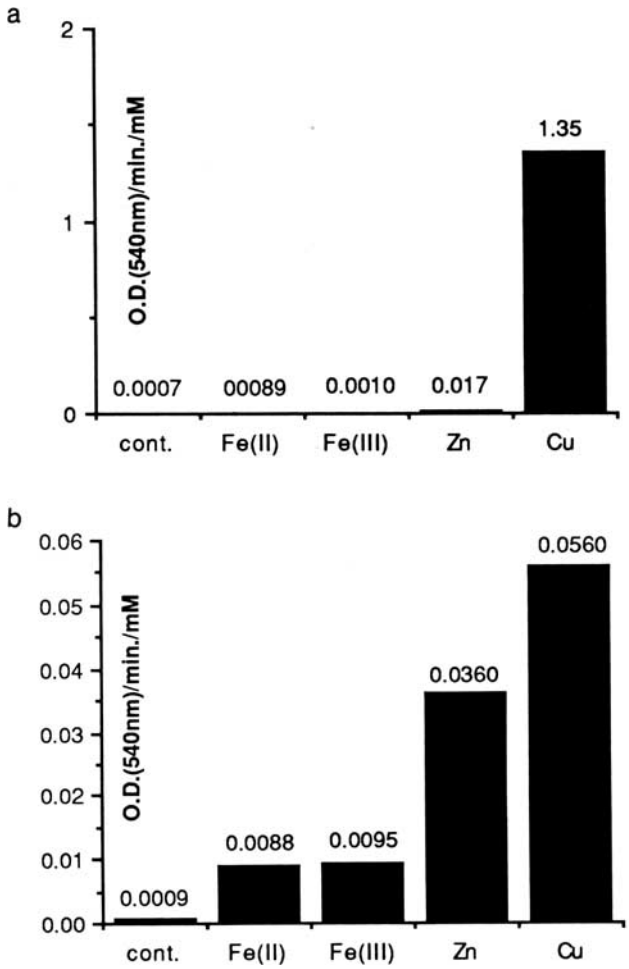


Figure 11. Study of melanochrome formation from DI *(a)* or DICA *(b)* after addition of FE^{++}, Fe^{+++}, Zn^{++}, and Cu^{++} salts. Cu^{++} had the highest activity of polymer formation among these metals. Cont., control; O.D., optical density.

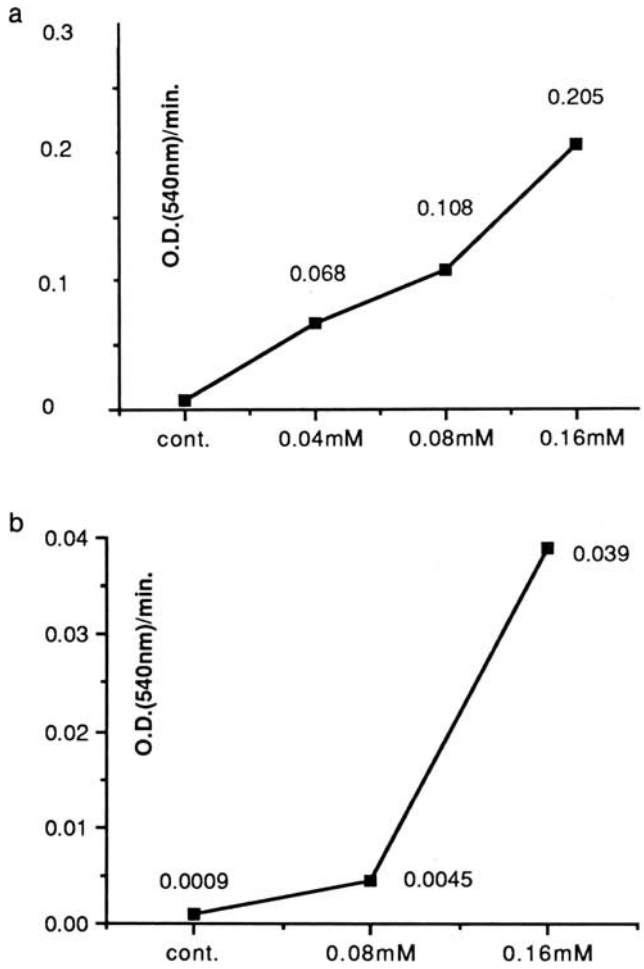


Figure 12. Result of melanin polymer formation assay from DI *(a)* or DICA *(b)* after addition of various concentration of Cu^{++}. When0.16 mM copper sulfate was added to the indole solution, DI exhibited a five times higher absorbance at 540 nm than DICA. Cont., control; O.D., optical density.

Prota [3] proposed that metal ions and, in particular, copper ions, enhance the formation of DICA, which is one of the most important indoles in the biosynthesis of melanin polymer. In natural eumelanin, the ratio of DICA to DI is higher than that in chemically formed eumelanin *in vitro*. In light of these advancements, many investigators have been trying to discern the proper function and distribution of metals in pigment cells. According to our microsensitive inductively coupled plasma mass spectrometry analysis, we found significant copper, iron, and zinc that varied in amount depending on the subcellular melanogenic compartments. For example, PMS were found to contain 20–30 times more copper than C V. Although copper ions are perhaps the most significant element for the enhancement of polymer formation, we also tried to enhance the formation of eumelanin polymer from monomers by the addition of hydrogen peroxide. We could, however, only accomplish the conversion from monomer to polymer by the introduction of copper ions, which led us to tentatively conclude that copper ions are one of the indispensable enhancing factors in eumelanin-polymer formation.

Two more significant factors in the regulation of melanogenesis that were recently investigated are dopachrome tautomerase and b-locus protein. King and coworkers and Korner and Pawelek reported the presence of an enzyme capable of converting dopachrome to DI or DICA, which they named dopachrome oxidere-ductase, or dopachrome conversion factor [19,20]. Recently, an enzyme independent from metals and specifically responsible for the conversion of L-dopachrome to DICA was determined to be dopachrome tautomerase [21–24].

The b-locus protein was discovered by chance when an anti-tyrosinase-4 monoclonal antibody named TMH-1 was cloned using a cDNA tyrosinase gene cloned by Shibahara *et al* [25]. Jackson recently reported, however, that the actual tyrosinase gene was found on the c-locus not the b-locus, as previously described [26].

Following this study, Halaban and Tomita and Montague investigated the function of the b-locus protein, which was related to tyrosinase, and found that the b-locus protein was actually gp75, which had catalase activity and was localized in MS [18,27].

We carried out immunoelectron microscopic analysis of the localization of the b-locus protein and found it partially in GERL and in some CV, PMS, and MS [28]. Moreover, the amount of b-locus found in PMS increases according to the maturation stage of the PMS. These findings suggest that the b-locus protein moves with tyrosinase and after its glycosylation becomes more potent, although its true function remains unclarified.

A summary of the intracellular localization of various regulatory factors involved in the process of melanogenesis is shown in Table III. With regard to melanin-polymer formation, catalase acts as the significant suppressor and is found most abundantly in CV, whereas both peroxidase and metals function as melanogenic enhancers within the PMS.

Table III. Intercellular Localization of Regulatory Factors Involved in Melanogenesis

	Ribosome	GERL	CV	PMS	MS
Tyrosinases	$Ty_2(+)$	$Ty_1(++)$	$Ty_1(+++)$	$Ty_3(++)$	$Ty_3(+-)$
Catalase	++	+	++	+	+
Peroxidase	–	–	+	++	+
Metals	ND[a]	ND	+	++	++
Monomer (DICA)	ND	ND	++	+	–
Melanin polymer	–	–	–	+	+++

[a]ND, not done.

Our present findings indicate that all processes leading to the terminal step of melanogenesis, namely, melanosomal melanin-polymer formation, are performed and controlled by regulatory factors in the subcellular melanogenic non-melanosomal compartments. These results should be further verified by the analysis of hypomelanocytic and hypermelanocytic dysfunction to initiate new therapeutic drug design.

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REFERENCES

1. Mishima Y: A post-melanosomal era: control of melanogenesis and melanoma growth (Presidential Address). Proceedings of the XIVth International Pigment Cell Conference in Kobe, Japan, 1990. Pigment Cell Res Suppl 2:3–16, 1992
2. Hatta S, Mishima Y: Melanin monomers within coated vesicles and premelanosomes in melanin synthesizing cells. J Invest Dermatol 91:181–184,1988
3. Prota G: Recent advances in the chemistry of melanogenesis in mammals. J Invest Dermatol 75:122–127, 1980
4. d'Ischia M, Napolitano A, Prota G: Peroxidase as an alternative to tyrosinase in the oxidative polymerization of 5,6-dihydroxyindoles to melanin(s). Biochim Biophys Acta 1073:423–430, 1991
5. Fahimi HD: Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). J Histochem Cytochem 16:547, 1968
6. Usami M, Takahashi A, Kadota K: Protein kinase and its endogenous substrates in coated vesicles. Biochem Biophys Acta 798:306–312, 1984
7. Imokawa G, Mishima Y: Isolation and biochemical characterization of tyrosinase-rich GERL and coated vesicle in melanin synthesizing cells. BrJ Dermatol 104:169–178, 1981
8. Lowry OH, Rosebrough NJ, Farr AI, Randoll RJ: Protein measurement with the Folin Phenol reagent. J Biol Chem 193:265–274, 1951
9. Chance B, Maehly AC: Assay of catalase and peroxidase; IV. Peroxidase assay by spectrophotometric measurements of the disappearance of hydrogen donor or the appearance of their colored oxidation products; [A] The guaiacol test. Methods Enzymol 11:770–773, 1969
10. Seiji M, Shimao K, Birbeck MSC, Fitzpatrick TB: Subcellular localization of melanin biosynthesis. Ann NY Acad Sci 100:497–533,1963
11. Mason HS: The chemistry of melanin, mechanism of the oxidation of dihydroyphenylalanine by tyrosinase. J Biol Chem 172:83–92, 1948
12. Mishima Y, Imokawa G, Ogura H: Functional and three-dimensional differentiation of smooth membrane structure in melanogenesis. In: Klaus SN (ed.). Pigment Cell, Vol. 4. Biologic Basis of Pigmentation. Karger, Basil, 1979, p 277–290
13. Imokawa G, Mishima Y: Loss of melanogenic properties in tyrosinase induced by glycosylation inhibitors within malignant melanoma cells. Cancer Res 42:1994–2002, 1982
14. Mishima Y, Imokawa G: Selective aberration and pigment loss in melanosomes of malignant melanoma cell *in vitro* by glycosylation inhibitors; premelanosomes as glycoprotein. J Invest Dermatol 2:81, 1983
15. Ito M: Studies on melanin (report II). Tohoku University, Sendai, 1957
16. Okun M: Peroxidase activity in normal and neoplastic melanocytes. J Invest Dermatol 48:461–465, 1967
17. Yamamoto H, Takeuchi S, Kudo T, Makino K, Nakata A, Shinoda T, Takeuchi T: Cloning and sequencing of mouse tyrosinase cDNA. Jpn J Genet 62:271–274, 1987
18. Halaban R, Moellman G: Murine and human b locus pigmentation genes encode a glycoprotein (gp75) with catalase activity. Proc Natl Acad Sci USA 87:4809–4813, 1990
19. Burber JI, Townsend D, Olds DP, King RA: Dopachrome oxidoreductase: a new enzyme in the pigment pathway. J Invest Dermatol 83:145–149, 1984
20. Korner A, Pawelek J: Mammalian tyrosinase catalyzes three reactions in melanin biosynthesis. Science 217:1163, 1982
21. Aroca P, Solano F, Garcia-BorronJC, Lozano JA: A new spectrophotometric assay for dopachrome tautomerase. J Biochem Biophys Methods 21:35–46, 1990
22. Townsend D, Oetting W, Polman T, King RA: Purification and characterization of dopachrome tautomerase (DT). Proceedings of the XIVth International Pigment Cell Conference, Kobe, Japan. Pigment Cell Res Suppl 2:32–35, 1992
23. Palumbo A, *et al*: Comparative action of dopachrome tautomerase and metal ions on the rearrangement of dopachrome (unpublished)
24. Pawelek JM: After dopachrome? Pigm Cell Res 4:53–62, 1991
25. Shibahara S, Tomita Y, Sakakura T, Nager C, Chandhuri B, Muller R: Cloning and expression of cDNA encoding mouse tyrosinase. Nucleic Acids Res 14:2413–2427, 1986
26. Jackson I: A cDNA encoding tyrosinase related protein maps to the brown locus in mouse. Proc Natl Acad Sci USA 85:4392–4396, 1988
27. Tomita Y, Montague PM, Hearing V: Anti-T_4 -tyrosinase monoclonal antibodies – specific markers for pigmented melanocytes. J Invest Dermatol 85:426–430, 1985
28. Shibata T: Localization of b-locus protein and melanin monomer in coated vesicles and premelanosomes of pigment cells. Workshop, WII-D6, Melanosome Proteins. Proceedings of the XIVth International Pigment Cell Conference in Kobe, Japan, 1990