Specific Incorporation of 4-*S*-Cysteinylphenol into Human Melanoma Cells*

Takuro Nakamura, M.D., Sachiko Seki, Osamu Matsubara, M.D., Shosuke Ito, Ph.D., and Tsutomu Kasuga, M.D.
Second Department of Pathology, School of Medicine, Tokyo Medical and Dental University (TN, SS, OM, TK), Tokyo, and School of Hygiene, Fujita-Gakuen Health University (SI), Aichi, Japan

The incorporation of 4-*S*-cysteinylphenol (4-S-CP), a tyrosine analog, into malignant melanoma cells was evaluated. 4-S-CP was specifically incorporated into the melanotic melanoma cells (HMV-II), which have activity for melanin synthesis, but was scarcely incorporated into HeLA S3 or HMV-I cells, which have no activity for melanin synthesis. Electron microscopic autoradiography revealed that the intracellular localization of 4-S-CP was closely correlated with melanogenesis and that 4-S-CP served as an initial substrate for tyrosinase and was utilized in melanin synthesis. On the basis of these findings we hypothesize that tyrosinase is required for intracellular incorporation of 4-S-CP. This specific incorporation of 4-S-CP into melanoma cells should be useful in the development of an effective procedure for chemotherapy of malignant melanomas and in analysis of melanin synthesis. *J Invest Dermatol 90:725–728, 1988*

Melanocytes or nevus cells have a special metabolic system for melanin synthesis promoted by the pigment-forming enzyme tyrosinase [1]. Malignant melanomas originate from melanocytes, and the melanin synthesis of these cells seems to increase greatly on their malignant transformation.

Several melanin precursors and their analogs are known to exhibit selective toxicity on malignant melanoma cells [2–5]. These compounds, which are believed to be substrates of tyrosinase, have been proposed as effective chemotherapeutic agents against malignant melanomas. Consistent with this idea, Prota [6] showed that 5-*S*-cysteinyl-3,4-dihydroxyphenylalanine (5-*S*-cysteinyldopa), a natural catecholic amino acid that is a building block of pheomelanins, exhibits antitumor activity against B-16 melanoma in mice.

In an attempt to obtain a new antitumor agent, Ito et al [2] synthesized 4-*S*-cysteinylphenol (4-S-CP), which is the *S*-homolog of L-3,4-dihydroxyphenylalanine (L-dopa) and a structural analog of 5-*S*-cysteinyldopa. This paper reports studies on the incorporation of this new compound, 4-S-CP, into human malignant melanoma cells and on its relation to melanin synthesis.

MATERIALS AND METHODS

Compound 4-S-CP was obtained by reaction of phenol with L-cystine in boiling aqueous HBr and was separated from 2-*S*-cysteinylphenol by fractional crystallization by one of the authors (SI) [2]. Tritiated 4-S-CP (specific activity 6.67 Ci/mmol) was produced by Amersham International Co. (Buckinghamshire, England).

Cells Two cell lines of human malignant melanoma were used: HMV-II cells [7], which are pigmented, and HMV-I cells [8], which are the nonpigmented counterpart of HMV-II, which do not synthesize melanin. HeLa S3 cells [9] originated from a human carcinoma of the uterine cervix. The culture media used were Ham's F-10 supplemented with 20% fetal bovine serum for HMV-II cells and Ham's F-10 supplemented with 10% bovine serum for HMV-I and HeLa S3 cells.

Quantitative Study of [^{3}H]4-*S*-Cysteinylphenol Incorporation into Culture Cells Inocula of 2×10^5 cells of each cell line were incubated in culture dishes at 37°C in 95% air–5% CO_2 for 48 h. Then [^{3}H]4-S-CP was added at 5 μCi/ml. After labeling for periods of 3–36 h, the cells were rinsed several times with fresh medium, collected on GF/C glass fiber filters, washed with cold phosphate buffer, and dried. Then they were counted in 10 ml of ACS-II scintillation fluid (Amersham International Co.) in a Packard Tri-Carb 460CD liquid scintillation spectrometer. Values for triplicate dishes were measured and expressed as the means ± SEM. Phenylthiourea (Sigma, St. Louis, MO) was added to control dishes to block tyrosinase [10,11].

Microautoradiography The cells were prepared in tissue culture chambers of 2 ml (Miles Scientific, Naperville, IL) and incubated at 37°C in 95% air–5% CO_2 for 48 h. After labeling with [^{3}H]4-S-CP at a concentration of 5 μCi/ml for 6 h, they were fixed in situ with methanol–acetate solution, coated with 2-fold diluted Sakura NR-M_2 emulsion (Konishiroku Photo Co., Tokyo) by dipping, dried, and kept at 4°C in the dark for 2 weeks. Then the films for autoradiography were developed with Konidol-X developer (Konishiroku Photo Co.), fixed in Fujifix (Fuji Photo Film Co., Tokyo), rinsed with running water, and lightly stained with Giemsa and hematoxylin solution.

Electron Microscopic Autoradiography Inocula of 1×10^6 HMV-II cells were incubated in tissue culture bottles at 37°C in 95% air–5% CO_2 for 48 h, and then [^{3}H]4-S-CP was added at a concentration of 5 μCi/ml. Pulse labeling was done for 1 h.

After subsequent incubation for 1, 24, or 192 h, cells in tissue culture bottles were rinsed several times with phosphate buffer, fixed with 2.5% phosphate-buffered glutaraldehyde at 4°C for 20 min and then 2% buffered osmium tetroxide at 4°C for 1 h, and

Manuscript received March 17, 1987; accepted for publication January 13, 1988.

*This work was supported in party by grants-in-aid for cancer research (58010030, 59010027, 6001003) and scientific research (60480144) from the Ministry of Education, Science and Culture of Japan.

Reprint requests to: Takuro Nakamura, M.D., Department of Pathology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 105, Japan.

Abbreviations:
L-dopa: L-3,4-dihydroxyphenylalanine
4-S-CP: 4-*S*-cysteinylphenol

0022-202X/88/$03.50

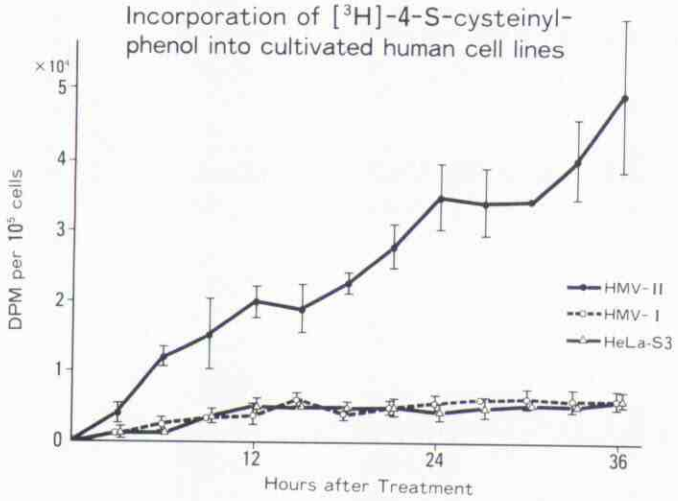


Figure 1. Radioactivity of cultured cells after labeling with [³H]4-S-CP.

dehydrated. The cells were collected after each step by low-speed centrifugation at 1500 rpm and were finally embedded in Epon 812.

Ultrathin sections were cut on a Porter-Blum ultramicrotome with sapphire knives (Sakura Seiki Co., Tokyo) and stained with 2% uranyl acetate for 20 min. The grid meshes with ultrathin sections were coated with 6-fold diluted Sakura NR-H_2 emulsion (Konishiroku Photo Co.) at 45°C, by the touching method, and kept in a dark box at 4°C for 5 weeks. The grids were developed with Elon ascorbic acid (EAA) for 15 min at 17°C, after gold intensification. After fixation with Fujifix, the meshes were stained with Mizuhira and Kurotaki's lead solution [12] for 15 min, rinsed, dried, and observed with a Hitachi H-500 electron microscope at 75 kV.

RESULTS

Selectivity and Time Dependence of Incorporation of [³H]4-S-CP [³H]4-S-CP was selectively incorporated into HMV-II melanotic melanoma cells. The radioactivity of HMV-II cells increased gradually with time, being about 5×10^4 dpm/1×10^5 cells after 36 h incubation (Fig 1). The radioactivities of HMV-I and HeLa S3 cells were much lower, being about 0.5×10^4 dpm/1×10^5 cells.

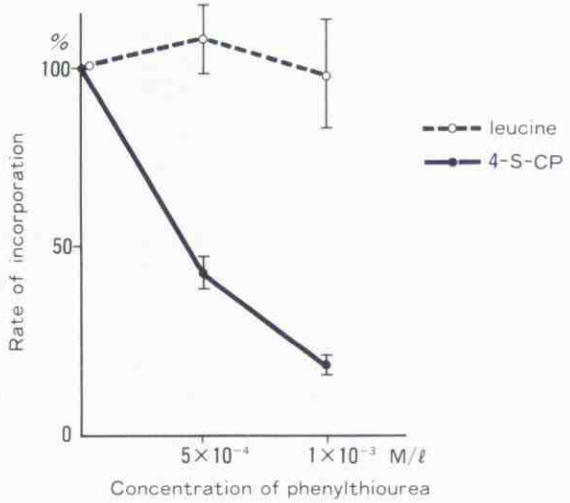


Figure 2. Inhibition of incorporation of [³H]4-S-CP into HMV-II melanoma cells by phenylthiourea. Incorporation of [³H]leucine into HMV-II cells was not suppressed by phenylthiourea. The time of incubation was 24 h, and radioactivity for the 100% control was 30,760 dpm per 10^5 cells.

In the presence of phenylthiourea, a copper-binding agent, the incorporation of [³H]4-S-CP into HMV-II cells was significantly inhibited (Fig 2).

Microautoradiographic Findings Silver grains were seen only on HMV-II cells. These grains were evenly distributed in the cytoplasm and accumulated on one side of the nucleus in a few cells (Fig 3*A*). No accumulation of grains on HMV-I cells (Fig 3*B*) or HeLa S3 cells (Fig 3*C*) was observed.

Electron Microscopic Autoradiographic Findings Table I shows the accumulations of silver grains in intracellular organelles

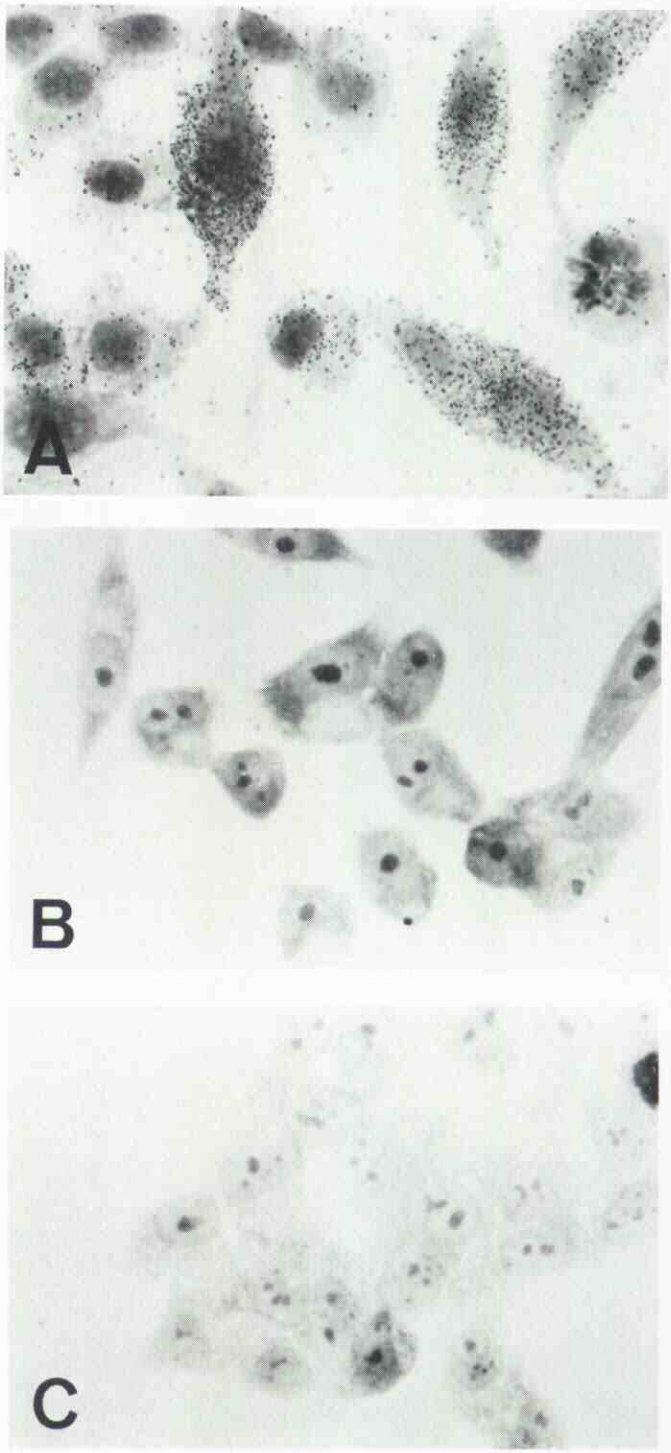


Figure 3. Microautoradiography of [³H]4-S-CP. *A*, HMV-II cells; *B*, HMV-I cells; *C*, HeLa S3 cells. Silver grains are seen only on HMV-II cells.

Table I Intracellular Distribution of [^{3}H]4-S-CP in HMV-II Cultured Melanoma Cells, 1, 24, and 192 Hours after Labeling[a]

Intracellular Organelles	Time Interval after Labeling (h)		
	1	24	192
Total number of grains	601	647	625
Cytoplasm[b]	77.5	75.9	82.6
Free ribosomes	58.8[c]	46.7[c]	31.7[c]
rER ribosomes	2.5	6.5	2.2
sER	3.8	0	8.0
Melanosomes	0	11.3[c]	20.2[c]
Mitochondria	5.0	0	16.8
Membrane	5.0	9.7	3.7
Golgi apparatus	2.5	1.7	0
Nucleus	22.5	24.1	17.4
Chromatin	20.0	19.3	11.9
Nucleolus	0	0	1.6
Membrane	2.5	4.8	3.9

[a] Data are expressed as percentages of the number of grains in each organelle to the total grain counts in 10–15 cells.
[b] rER, rough endoplasmic reticulum; sER, smooth endoplasmic reticulum.
[c] The decrease in labeling of free ribosomes between 1 and 24 h and between 24 and 192 h is statistically significant ($p < 0.001$). The increase in labeling of melanosomes between 24 and 192 h is also significant ($p < 0.001$). Chi-square test with Yates' correction was used for statistical evaluation.

1, 24, and 192 h after labeling. The silver grains were found in the various intracellular organelles and in the nucleus (Fig 4*A*). Initially most grains were associated with free ribosomes (Fig 4*B*), but later they became incorporated into melanosomes and premelanosomes (Fig 4*A*). There were only a few silver grains in the rough endoplasmic reticulum and Golgi apparatus.

DISCUSSION

Melanocytes are unique in possessing a copper enzyme, tyrosinase, which catalyzes the oxidation of tyrosine to dopa and then the oxidation of dopa to melanin. This enzymatic activity is thought to be useful for therapy by selective toxicity of melanin analogs and for analysis of melanogenesis. 4-S-CP is structurally similar to tyrosine, differing only in containing a sulfur atom (Fig 5), and it is thought to be oxidized by tyrosinase and molecular oxygen to 4-*S*-cysteinyl-catechol and orthoquinone form, in the same way as tyrosine is oxidized to L-dopa and dopa-quinone.

The incorporations of [^{3}H]tyrosine and [^{3}H]L-dopa into malignant melanoma cells were studied, and the latter was found to be incorporated selectively [8,13]. 4-S-CP is incorporated into melanoma cells more rapidly than tyrosine. The following explanations can be proposed for this phenomenon: (1) 4-S-CP resembles tyrosine structurally and can serve as a substrate for tyrosinase [2]; (2) tyrosine is used extensively in general cellular protein synthesis [13], but there is the possibility that 4-S-CP might not be used for the synthesis of other proteins because it is not a natural amino acid for protein synthesis. HMV-II cells are rich in melanin and give a positive reaction for dopa, whereas HMV-I cells do not contain melanin or premelanosomes and give a negative reaction for dopa. These facts and the finding that phenylthiourea, a tyrosinase inhibitor, inhibited the incorporation of 4-S-CP into HMV-II cells indicate that tyrosinase is required for the incorporation of 4-S-CP.

Malignant melanomas respond poorly to usual chemotherapy treatments, so their selective chemotherapy by procedures based on the unique enzymatic process of conversion of tyrosine to melanin in melanocytes seems important. There are many reports [2–5,14–18] of the toxicity of tyrosine metabolites in cells that synthesize melanin and on selective chemotherapy, but so far this therapeutic procedure has not been very effective.

However, there is evidence that 4-S-CP and related compounds are effective against malignant melanomas. Jimbow et al [19] reported that systemic administration of 4-S-CP increased the survival time of B-16 melanoma-bearing mice. Moreover, 4-S-CP and other derivatives that serve as substrates of tyrosinase were reported to have a therapeutic effect against human malignant melanoma cells [20].

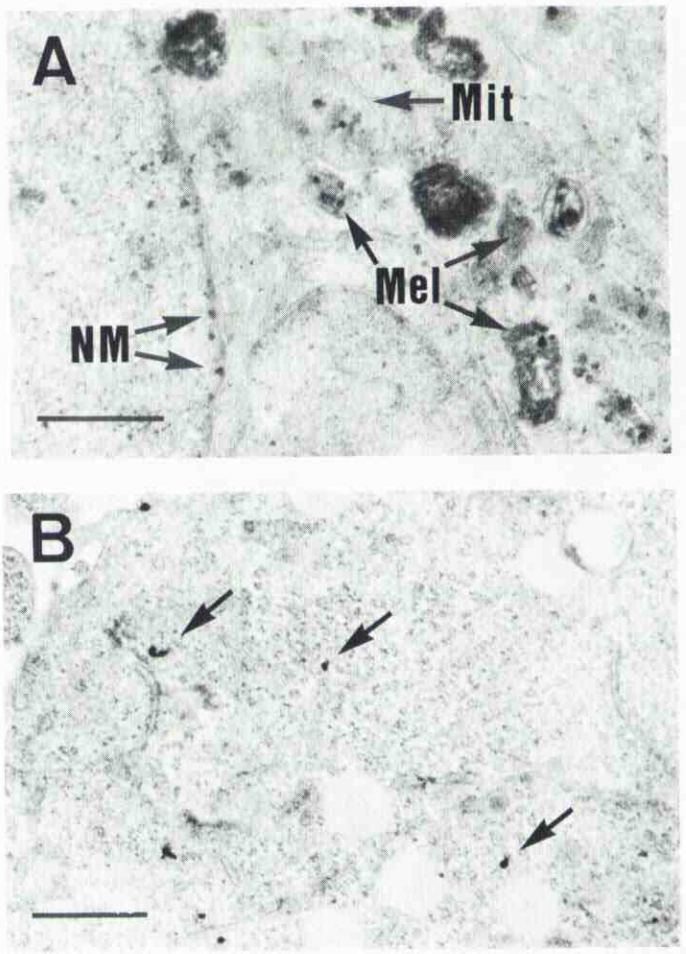
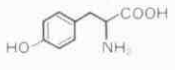


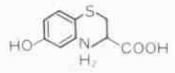
Figure 4. Electron microscopic autoradiography of [^{3}H]4-S-CP. *A*, Silver grains on many melanosomes *(Mel)*. Grains are also seen on the nuclear membrane *(NM)* and mitochondria *(Mit)* (192 h after labeling). *B*, Silver grains on free ribosomes *(arrows)* (1 h after labeling). *Bars* = 1 μm *(A)* and 0.5 μm *(B)*.

There is much morphological information on melanosome formation [21–23], but several hypotheses have not yet been confirmed. Tyrosinase is said to be transferred from the rough endoplasmic reticulum to the Golgi apparatus, and then a small vacuole formed in the Golgi cisterna becomes a melanosome [24].

Novikoff et al [25] proposed the "GERL" hypothesis: that the premelanosomes arise from dilated cisternae of endoplasmic reticulum, generally in a specialized region near the Golgi apparatus. Likewise, Maul [21] postulated that most premelanosomes are present in and develop within a tubular smooth endoplasmic reticulum. Kasuga et al [8] determined the intracellular localizations of [^{3}H]tyrosine and [^{3}H]L-dopa by electron microscopic autoradiography. [^{3}H]4-S-CP is a more specific marker of melanogenesis than



TYROSINE



4-S-CYSTEINYL-PHENOL

Figure 5. Structures of tyrosine and 4-S-CP.

[^{3}H]L-dopa. We found that 4-S-CP was initially present mainly in free ribosomes and then gradually became located in melanosomes. The rough endoplasmic reticulum and the Golgi apparatus seemed to have little relation to the incorporation of 4-S-CP.

With the availability of tyrosine analogs that have stronger cytotoxicity than 4-S-CP [20], it should be valuable as a targeted "prodrug" in development of a rational chemotherapy of malignant melanoma. In this study we demonstrated a close correlation between 4-S-CP and melanin synthesis. Because of this finding, we could obtain data on the location of tyrosinase activity and the route of transfer of metabolites.

The authors thank Professor K. Ishikawa, Department of Biochemistry, School of Medicine, Yamagata University, for helpful discussion and suggestions.

REFERENCES

1. Fitzpatrick TB, Becker SW Jr, Lerner AB, Montgomery H: Tyrosinase in human skin: demonstration of its presence and of its role in human melanin formation. Science 112:223–225, 1950
2. Ito S, Inoue S, Yamamoto Y, Fujita K: Synthesis and antitumor activity of cysteinyl-3,4-dihydroxyphenylalanine and related compounds. J Med Chem 24:673–677, 1981
3. Wick MM, Byers L, Frei E III: L-Dopa: selective toxicity for melanoma cells in vitro. Science 197:468–469, 1977
4. Wick MM: An experimental approach to the chemotherapy of melanoma. J Invest Dermatol 74:63–65, 1980
5. Wick MM, Byers C, Ratkliff J: Selective toxicity of 6-hydroxydopa for melanoma cells. J Invest Dermatol 72:67–69, 1979
6. Prota G: Recent advances in the chemistry of melanogenesis in mammals. J Invest Dermatol 75:122–127, 1980
7. Kasuga T, Nojiri I, Furuse K, Kubo E, Noda Y, Sugano H, Sakamoto G: Establishment of human melanoma cell like (HMV-II). Proceedings of the 35th Annual Meeting of Japanese Cancer Society, 1976, 102 pp (in Japanese)
8. Kasuga T, Furuse T, Takahashi I, Tsuchiya E: Ultrastructural and autoradiographic studies on melanin synthesis and membrane system using cultured B16 melanoma, irradiated melanoma, and human malignant melanoma. In: Biology of Normal and Abnormal Melanocytes. Edited by T Kawamura, TB Fitzpatrick, and M Seiji. University of Tokyo Press, Tokyo, 1971, pp 241–264
9. Puck TT, Marcus PI, Cierciura S: Clonal growth of mammalian cells in vitro. Growth characteristics of colonies from single HeLa cells with and without a "feeder" layer. J Exp Med 103:273–284, 1956
10. Lerner AB, Fitzpatrick TB: Biochemistry of melanin formation. Physiol Rev 30:91–126, 1950
11. Morrison ME, Yagi MJ, Cohen G: In vitro studies of 2,4-dihydroxyphenylalanine, a prodrug targeted against malignant melanoma cells. Proc Natl Acad Sci USA 82:2960–2964, 1985
12. Mizuhira V, Shiihashi M, Futaesaku Y: High-speed electron microscope autoradiographic studies of diffusible compounds. J Histochem Cytochem 29:143–160, 1981
13. Wick MM, Frei E III: Selective incorporation of L-3,4-dihydroxyphenylalanine by S-91 Cloudman melanoma in vitro. Cancer Res 37:2123–2125, 1977
14. Graham DG, Tiffany SM, Vogel FS: The toxicity of melanin precursors. J Invest Dermatol 70:113–116, 1978
15. Fujita K, Ito S, Inoue S, Yamamoto Y, Takeuchi J, Shamoto M, Nagatsu T: Selective toxicity of 5-*S*-cysteinyldopa, a melanin precursor, to tumor cells in vitro and in vivo. Cancer Res 40:2543–2546, 1980
16. Wick MM: L-Dopa methyl ester as a new antitumor agent. Nature 269:512–513, 1977
17. Wick MM: L-Dopa methyl ester: prolongation of survival of neuroblastoma-bearing mice after treatment. Science 199:775–776, 1978
18. Wick MM: Dopamine: a novel antitumor agent active against B-16 melanoma in vivo. J Invest Dermatol 71:163–164, 1978
19. Jimbow K, Miura S, Ito K, Kasuga T, Ito S: Utilization of melanin precursors for experimental chemotherapy of malignant melanoma. Jap J Cancer Chemother 11:2125–2132, 1984 (in Japanese)
20. Yamada I, Seki S, Matsubara O, Ito S, Suzuki S, Kasuga T: The cytotoxicity of cysteinylcatechols and related compounds to human melanoma cells in vitro. J Invest Dermatol 88:538–540, 1987
21. Maul GG: Golgi–melanosome relationship in human melanoma in vitro. J Ultrastruct Res 26:163–176, 1969
22. Seiji M: Melanin metabolism in the skin. Ann Jap Dermatol 88:763–789, 1978 (in Japanese)
23. Ogura H, Mishima Y: GERL-coated vesicle system in the melanin synthesizing cell: its three-dimensional ultrastructure and tyrosinase activity. Ann Jap Dermatol 87:883–901, 1977 (in Japanese)
24. Seiji M, Iwashita S: Intracellular localization of tyrosinase and site of melanin formation in melanocyte. J Invest Dermatol 45:305–314, 1965
25. Novikoff AB, Albala A, Biempica L: Ultrastructural and cytochemical observations on B-16 and Harding Passey mouse melanomas. The origin of premelanosomes and compound melanosomes. J Histochem Cytochem 16:299–319, 1968