

Production, Circulation, and Excretion of Melanin-related Metabolites in B16 Melanoma-bearing Mice

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Urinary 5-S-cysteinyl-dopa (5-S-CD) has been used as a biochemical marker of melanoma metastasis. A method was developed for determining the eumelanin-related metabolites 5(6)-hydroxy-6(5)-methoxyindole-2-carboxylic acids (5H6MI2C and 6H5MI2C) in small volumes of serum. We compared these indoles and 5-S-CD regarding the correlation of their production in melanoma, circulation in blood, and excretion in urine, with the weight of highly pigmented, B16 mouse melanoma. An excellent correlation was found between the serum concentration of 5H6MI2C + 6H5MI2C ($r = 0.92$) and 5-S-CD ($r = 0.89$) and tumor weight. However, the urinary excretion of 5H6MI2C + 6H5MI2C and 5-S-CD did not show any significant correlation. These results suggest that 5H6MI2C + 6H5MI2C and 5-S-CD in serum may better reflect melanoma progression than those in urine. Furthermore, comparison of the contents of these melanin-related metabolites between highly pigmented and less pigmented B16 melanomas suggests that 5-S-CD may be accumulated in pigmented melanoma by virtue of binding to melanin and that catechol-O-methyltransferase (COMT) may play a regulatory role in pigmentation. **Key words:** 5(6)-Hydroxy-6(5)-methoxyindole-2-carboxylic acids; 5-S-Cysteinyl-dopa; Melanoma.

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Malignant melanoma is a unique tumor that produces the pigment melanin. Tyrosinase, an enzyme specific for melanocytes, catalyses the oxidation of the amino acid tyrosine to dopa and then to dopaquinone. The spontaneous reaction of dopaquinone leads to the formation of 5,6-dihydroxyindole (5,6DHI) and 5,6-dihydroxyindole-2-carboxylic acid (5,6DHI2C) (1). Although this reaction can proceed non-enzymically, factors that accelerate or modify it have been found (2). These indoles are then further oxidised eventually to produce the brown-black pig-

ment eumelanin (3). On the other hand, in the presence of cysteine (or glutathione), a rapid reaction with dopaquinone gives 5-S-cysteinyl-dopa (5-S-CD) along with minor isomers (1). Oxidation of cysteinyl-dopas leads to the reddish-brown pigment pheomelanin.

Among these melanin precursors, 5-S-CD has been most extensively used as a biochemical marker of melanoma progression. Many reports have shown that urinary 5-S-CD is increased in patients with metastatic melanoma (4, 5). Plasma levels of 5-S-CD have also been shown to reflect melanoma progression in human subjects (6) as well as in B16 melanoma-bearing mice (7). On the other hand, the eumelanin precursors 5,6DHI and 5,6DHI2C are not detected in urine in the free forms, as they are extremely labile to autoxidation and they are readily metabolised to the O-methyl derivatives and their glucuronide and sulfate conjugates (8, 9). It has been known for some years that high levels of these eumelanin-related metabolites are also excreted in the urine of melanoma patients (9, 10). Among the eumelanin-related metabolites, the two O-methyl derivatives of 5,6DHI2C, viz. 5(6)-hydroxy-6(5)-methoxyindole-2-carboxylic acids (5H6MI2C and 6H5MI2C) appear to be suitable as biochemical markers because they are stable and occur mainly in the free forms, in contrast to the O-methyl derivatives of 5,6DHI (8).

In previous studies, we have examined which of the two markers, 5-S-CD and the eumelanin-related metabolites, better reflect the progression of B16 mouse melanoma (11, 12). In this study, we developed a method to determine the indoles 5H6MI2C and 6H5MI2C in small volumes of serum and compared 5-S-CD and these indoles regarding their production in melanoma tissues, circulation in blood, and excretion in urine of B16-melanoma-bearing mice. We also compared differences in the contents of these melanin-related metabolites between highly pigmented and less pigmented B16 melanomas.

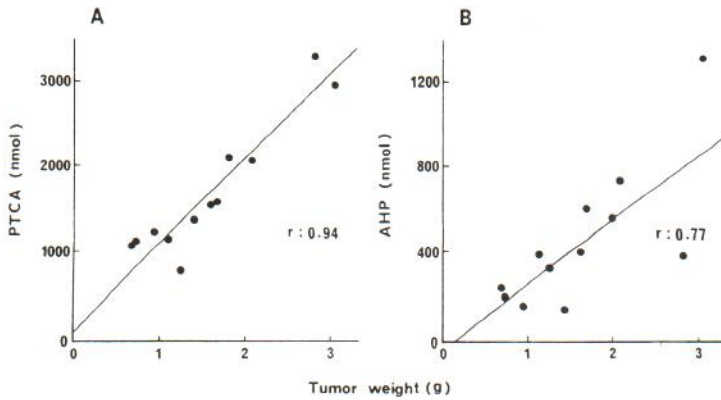


Fig. 1. Correlation between tumor weight and content of PTCA (A) and AHP (B).

MATERIALS AND METHODS

Animal experiments

A highly pigmented cell line of B16 melanoma was obtained from Menard Cosmetics Co., Ltd. 6 months before starting this study. A less pigmented cell line came from Sapporo Medical College in 1985. The latter gradually lost pigmentation during *in vivo* serial passages. The same cell line was used in the two previous studies (11, 12). B16 melanomas were maintained by subcutaneous inoculation of tumor cells on the back of C57BL/6 mice. Suspensions of melanoma cells were prepared by homogenisation of the excised tumors in phosphate-buffered saline and passed through a sterile 80-mesh stainless steel screen. The suspension of melanoma cells at a concentration of 5×10^6 cells in 0.5 ml was inoculated subcutaneously into the axillary region of male C57BL/6 mice (5 weeks old).

Animals were killed between days 21 and 28. Blood was collected from axillary fossa under anesthesia and the tumor was then excised and weighed. One day before killing, each mouse was kept in a metabolic cage and urine was collected for 24 h in a beaker containing 1 ml of 20% acetic acid and 20 mg of sodium metabisulfite.

High-performance liquid chromatography (HPLC)

The standards 5-S-CD, 5,6DHI2C, 5H6MI2C, and 6H5MI2C were prepared chemically as described by us elsewhere (13, 14). Indoles in urine and melanoma samples were determined by direct injection of urine samples or tumor extracts. Urine samples were centrifuged and 10- μ l aliquots were injected into the HPLC system. Melanoma tissues were homogenised with 9 volumes of 0.4 M HClO₄, centrifuged, and injected. The HPLC conditions were similar to those described by us (11, 12); an EICOM EC-100 electrochemical detector was used at +400 mV, vs. an Ag/AgCl electrode.

5-S-CD in serum and urine samples was determined after alumina extraction (15) and 5-S-CD in melanoma extracts by direct injection. The HPLC conditions were similar to those described by us (15); the pH of the mobile phase was adjusted to 3.10, and a JASCO 840-EC electrochemical detector was used at +750 mV.

Extraction of the indoles from serum

In a micro test tube (1.5-ml volume) were placed 20 mg of sodium chloride and 20 μ l of 1 M hydrochloric acid. 100 μ l of serum was added to the tube, and the tube was shaken briefly. The mixture was extracted twice with 1 ml of ethyl acetate by shaking for 2 min on a JASCO MT-30 multi-tube mixer. After centrifugation, the ethyl acetate layer was transferred to a test tube (20 ml) and concentrated in a Taiyo TC-10D test-tube concentrator under reduced pressure on the aspirator at 40°C. The residue was dissolved in 100 μ l of the mobile phase for HPLC analysis and transferred to a micro test tube. After centrifugation, 50 μ l of the supernatant was injected for HPLC.

Addition recovery and reproducibility of the extraction were estimated by adding 20 ng each of the indoles to a pooled human serum and analysing 8 aliquots at the same time.

Eu- and pheomelanin assay

Melanin contents were analysed by our HPLC method (16). Eumelanin was assayed by pyrrole-2,3,5-tricarboxylic acid (PTCA) after permanganate oxidation and pheomelanin was as aminohydroxyphenylalanine (AHP) after hydriodic acid hydrolysis.

RESULTS

Extraction of the indoles from serum

Preliminary experiments showed that under neutral conditions recoveries of 5H6MI2C and 6H5MI2C from serum were approx. 10%. But the addition of 1 M hydrochloric acid and sodium chloride greatly improved the recovery. Under these conditions, addition recoveries for 5H6MI2C and 6H5MI2C were 52 ± 6.3 and $71 \pm 6.8\%$, respectively. The serum contents of these indoles in this study were corrected for the recoveries. Although 5,6DHI2C could also be extracted from serum, its recovery was very low (approx. 20%).

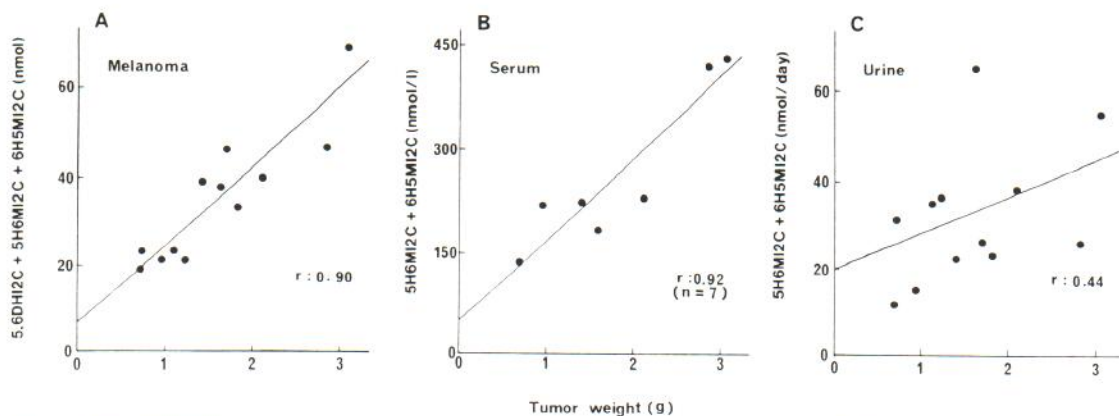


Fig. 2. Correlation between tumor weight and content of indoles in melanoma (A), serum (B), and urine (C). Five serum samples could not be analysed because of the limited quantity.

Correlation of production, circulation, and excretion of melanin-related metabolites with tumor weight

The contents of eumelanin (PTCA) and pheomelanin (AHP) in B16 melanoma tissues were analysed. An excellent correlation was found between PTCA content and tumor weight (Fig. 1A). The average content of PTCA was 1080 nmol (214 μg)/g tumor, which was roughly equal to 1.1 (w/w)% as eumelanin (16). AHP content showed a much weaker correlation with tumor weight (Fig. 1B). The average AHP content of 447 nmol (56 μg)/g tumor corresponded to 0.028 (w/w)% as pheomelanin.

5,6DHI2C, 5H6MI2C, and 6H5MI2C were detected in melanoma tissues; 5,6DHI2C was a chief indole, constituting 97% of the three indoles combined. 5,6DHI2C was also present in serum, but the exact concentration could not be determined due to

the low recovery. On the other hand, 5,6DHI2C was not detected in urine. The molar ratios of 5H6MI2C/6H5MI2C were $0.82 \pm 0.14:1$ ($n = 7$) in serum, but $3.2 \pm 0.77:1$ ($n = 12$) in urine.

The contents of indoles in melanoma and in serum correlated strongly with tumor weight (Fig. 2A, B). There was also a strong correlation between serum 6H5MI2C and tumor weight ($r = 0.93$). Correlation of indoles was not significant in urine (Fig. 2C).

Serum 5-S-CD showed a good correlation with tumor weight (Fig. 3B), while correlation was modest in melanoma (Fig. 3A). Urinary 5-S-CD did not show a significant correlation (Fig. 3C).

5-S-CD in melanoma had a six-fold greater concentration than have indoles, while the latter had serum concentration and urinary excretion rates 1.8 and 1.5 times greater than 5-S-CD (Table I). The ratios of urinary excretion/serum concentration

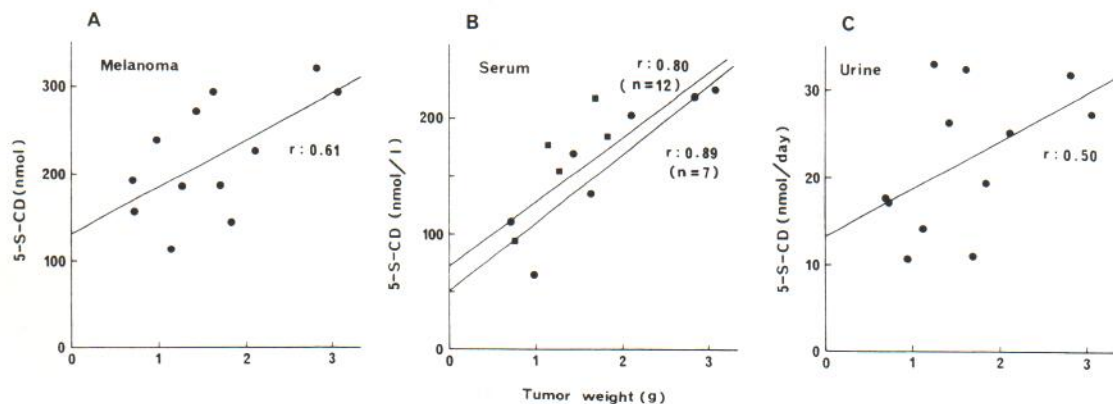


Fig. 3. Correlation between tumor weight and content of 5-S-CD in melanoma (A), serum (B), and urine (C). Correlation in serum was calculated for the seven samples that matched those in Fig. 2 (●) or for all of the twelve samples (● + ■).

Table I. Concentrations and ratios of indoles and 5-S-CD in melanoma, in serum, and in urine

Sample	Concentration	n	Indoles (5H6MI2C + 6H5MI2C)	5-S-CD	Indoles/5-S-CD ratio
Melanoma	nmol/g tumor	12	23±5 ^a	154±63	0.16±0.05
Serum	nmol/l/g tumor	7	155±42	95±31	1.76±0.79
Urine	nmol/day/g tumor	12	22±11	15±7	1.52±0.61

^a5,6DHI2C + 5H6MI2C + 6H5MI2C.

(clearance) were 138 ± 102 ml/day for indoles and 158 ± 40 ml/day for 5-S-CD, respectively ($n = 7$).

Comparison of highly pigmented and less pigmented melanomas

The B16 melanoma used in this study was highly pigmented, as evidenced by the high PTCA value of 1080 nmol/g tumor. In the two previous studies (11,12), less pigmented B16 melanoma was used, which had a PTCA value of 30 nmol/g tumor (Fig. 4A). These two variants of B16 melanoma cell lines showed distinctive biochemical features: 5,6DHI2C was present mostly in the free, non-O-methylated

form in highly pigmented melanoma, whereas less pigmented melanoma contained 5,6DHI2C, 5H6MI2C, and 6H5MI2C in a comparable ratio (Fig. 4A). The contents of indoles in melanoma tissues were at the same level (Fig. 4A) but the value of urinary excretion was four times greater in less pigmented melanoma than in highly pigmented melanoma (Fig. 4B).

The difference between melanoma content and urinary excretion was more pronounced in 5-S-CD. The content of 5-S-CD in highly pigmented melanoma was eight times greater than in less pigmented melanoma, while the value of urinary excre-

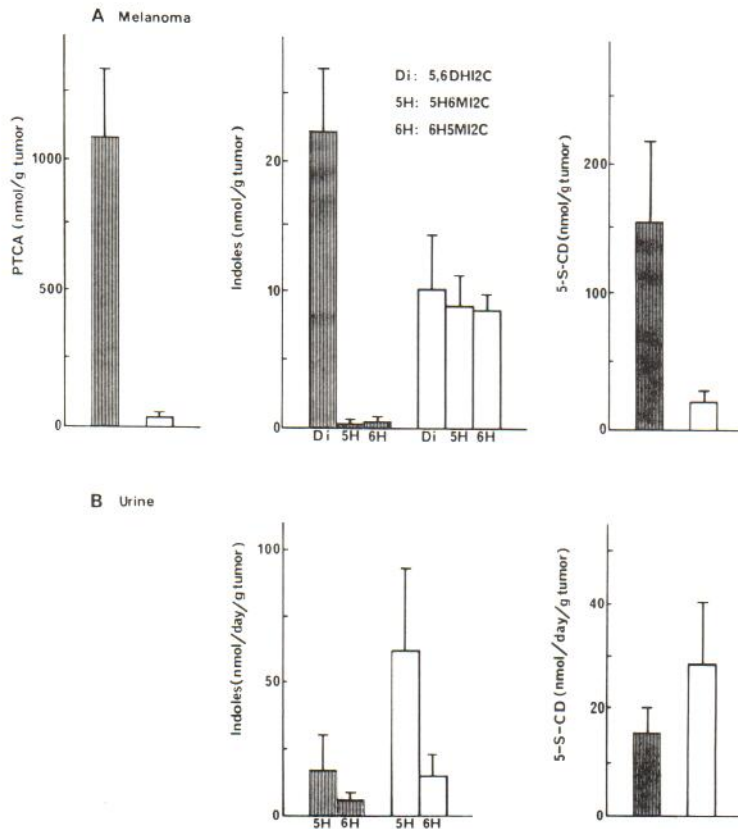


Fig. 4. (A) Contents of PTCA, indoles, and 5-S-CD in melanoma tissues. (B) Urinary excretion of indoles and 5-S-CD. ■, Highly pigmented melanoma ($n = 12$); □, less pigmented melanoma ($n = 9$).

tion was about one-half. The ratio of urinary excretion/melanoma content was thus 0.10/day in highly pigmented melanoma but 1.4/day in less pigmented melanoma.

DISCUSSION

Urinary 5-S-CD has been studied extensively as a marker of melanoma progression (4,5) and of degree of skin pigmentation, while plasma or serum 5-S-CD has also attracted attention by several research groups (6,7,17,18). Urinary excretion of eumelanin-related metabolites has also been shown to reflect melanoma progression (9–11) and pigmentation in skin (19,20). In the present study we have developed a method to determine the eumelanin-related indoles 5H6MI2C and 6H5MI2C using small volumes of serum.

We have analysed the contents of melanins in melanoma and melanin-related metabolites in melanoma, serum, and urine of B16-melanoma-bearing mice. Eumelanin (PTCA) content is closely correlated with tumor weight (Fig. 1A), which tallies with the fact that B16 melanoma produces almost pure eumelanin. Comparison of serum and urine as a specimen indicates that the contents of indoles (5H6MI2C + 6H5MI2C) and 5-S-CD in serum are much better correlated with tumor size than are those in urine. The serum concentration of indoles was 1.8 times greater than that of 5-S-CD. Plasma 5-S-CD level has recently been shown to be correlated with the size of B16 tumors (7). The present results suggest that serum 5H6MI2C + 6H5MI2C may also serve as a marker of melanoma progression. However, the combination of two or more markers would be more informative and reliable, especially in human subjects who have wide variations in biochemical features of melanoma and metabolic activities.

The ratio of 5H6MI2C/6H5MI2C showed a marked difference between serum and urine. Although the exact biochemical ground for this difference is unknown, it should be noted that 6H5MI2C is found to be more easily metabolised to the sulfate (12) and appears to be more easily oxidised.

B16 melanoma used in this study was highly pigmented, while in the previous studies (11,12) less pigmented B16 melanoma was used. We have no explanation why the latter gradually lost pigmentation during *in vivo* passages. Comparison of these two B16 melanomas suggests that melanin-related

metabolites, especially 5-S-CD, accumulate in the highly pigmented melanoma tissues (Fig. 4A). Melanin has been shown to have the properties of a polyanion and binds drugs that are positively charged at physiological pH (21). Thus, it is conceivable that a certain concentration of 5-S-CD formed in melanoma cells is trapped in the highly melanised melanosomes. This would explain the more than 10-fold increase in the ratio of total indoles/5-S-CD by diffusion into blood (Table I).

Finally, a role of COMT in pigmentation is suggested. One of the marked differences between the highly pigmented and less pigmented melanomas was the almost complete lack of O-methylation of 5,6DHI2C in the former (Fig. 4). This would help 5,6DHI2C integrate into the melanin being formed. Axelrod & Lerner (22) found that O-methylation of dihydroxyindoles was catalysed by COMT present in hamster melanoma and suggested that COMT may play a regulatory role in pigmentation. The same suggestion has been made also by Pavel et al. (8).

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