Melanin-related Metabolites in Urine of B16 Melanoma-bearing Mice

KAZUMASA WAKAMATSU, SHOSUKE ITO and KEISUKE FUJITA

School of Hygiene and Institute for Comprehensive Medical Science, Fujita-Gakuen Health University, Japan

Wakamatsu K, Ito S, Fujita K. Melanin-related metabolites in urine of B16 melanoma-bearing mice. Acta Derm Venereol (Stockh) 1988; 68: 385–389.

5-S-Cysteinyldopa (5-S-CD), a pheomelanin precursor, has been used as a biochemical marker of melanoma metastasis. Recently, 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI2C), a eumelanin-related metabolite, was shown to reflect well the degree of skin pigmentation. We measured the urinary excretion of 5H6MI2C and 5-S-CD in mice bearing B16 melanoma to determine which of the two markers better reflects the progression of melanoma. The urinary excretion of both 5H6MI2C and 5-S-CD increased rapidly in parallel with the tumour volume. The highest values for the two metabolites in melanoma-bearing mice were three orders of magnitude higher than those in control mice. However, 5H6MI2C had a higher excretion level at the early stage of melanoma progression, while 5-S-CD had a higher excretion level at the later stage. *Key words: 5-Hydroxy-6-methoxyindole-2-carboxylic acid; 5-S-Cysteinyldopa.* (Received Jan 26, 1988.)

S. Ito, School of Hygiene, Fujita-Gakuen Health University, Toyoake, Aichi 470-11, Japan.

Melanocytes possess a unique biochemical feature, melanogenesis. In melanocytes, a specific enzyme tyrosinase converts tyrosine to dopa and then to dopaquinone which is cyclyzed and oxidized to give rise to the brown-black eumelanin with the intermediacy of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid. If dopaquinone encounters cysteine or glutathione, the reddish-brown pheomelanin is produced via cysteinyldopas, among which 5-S-cysteinyldopa (5-S-CD) is the major isomer (1). These melanin precursors and their metabolites are detected in normal urine at low levels and in melanotic urine at high levels (2–7). Among these compounds, 5-S-CD has been most extensively studied as a biochemical marker of melanoma progression (8). However, recent studies by Pavel's group have indicated that the O-methyl derivatives of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid may also serve the same purpose (4). They have also shown that among dopa, 5-S-CD, 5-hydroxy-6-methoxyindole (5H6MI), and 5-hydroxy-6-methoxyindole-2-carboxylic acid (5H6MI2C), 5H6MI2C is the best urinary marker of melanin formation in normal skin pigmentary system (5).

In order to determine which of the two markers, 5H6MI2C and 5-S-CD, better reflects the progression of melanoma, we studied the urinary excretion of these metabolites in mice bearing B16 melanoma. Dopa, dopamine, and 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C) were also analysed for comparison.

MATERIALS AND METHODS

Chemicals

5-S-CD, 5H6MI2C, and 6H5MI2C were prepared chemically as described by us (9, 10). All other chemicals were of analytical grade and purchased either from Sigma Chemical Co. (St. Louis) or from Wako Pure Chemical Industries (Osaka).

Melanoma inoculation

B16 melanomas were maintained by subcutaneous inoculation of tumour cells on the back of C56BL/6 mice. Suspension of melanoma cells was prepared by homogenisation of the excised tumours 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 per 0.5 ml was inoculated subcutaneously into axillary region of 8 male C57BL/6 mice (5 weeks old, approx. 20 g). Two mice were used for control purposes. The tumour volume was calculated by the formula: long axis $\times$ (short axis)$^2\times1/2$.

Urine collection

24-h urine was collected 3 days before inoculation and every 3 days thereafter. Each mouse was kept in a metabolic cage and urine was collected in a beaker containing 1 ml of 20% acetic acid and 20 mg of sodium metabisulfite.

High-performance liquid chromatography (HPLC)

Dopa, dopamine, and 5-S-CD in urine samples were determined *ad modum* Ito et al. (11). 5H6MI2C and 6H5MI2C were determined fluorimetrically (12) as follows. Urine samples were centrifuged at 3000 rpm for 10 min and 10-μl aliquots were directly injected into the following HPLC system.

The HPLC system consisted of a Yanaco model L-2000 liquid chromatograph (Yanagimoto, Kyoto), a Yanaco ODS-A reversed-phase column (4.6×250 mm, 7 μm particle size), and a JASCO 820-FP spectrofluorimeter (Tokyo). The excitation and emission wavelengths were set at 315 and 390 nm, respectively (12). The mobile phase was 0.1 M potassium phosphate buffer, pH 2.1, containing 1 mM Na_2EDTA : methanol, 88 : 12 (v/v). The column was maintained at 50°C. The flow rate was 1.0 ml/min. Under these HPLC conditions, 5H6MI2C and 6H5MI2C appeared at 24 and 38 min, respectively.

RESULTS

Fig. 1 shows typical chromatograms of urine samples from a mouse before melanoma inoculation and 15 days after inoculation. In the control urine, 5H6MI2C and 6H5MI2C were barely detectable (detection limits: approx. 0.2 nmol/day for 5H6MI2C and 0.05 nmol/day for 6H5MI2C). Attempts to shorten the retention times resulted in interference by other peaks, thus giving artificially higher values for control urine. Melanoma-bearing mice excreted large amounts of 5H6MI2C and 6H5MI2C. Identification of these indoles was confirmed by comparing emission spectra of the peaks with those of the standards.

Fig. 2 and Table I show changes in the urinary excretion of 5H6MI2C and 5-S-CD in melanoma-bearing mice and the tumour volume. Tumours became palpable 9 days after inoculation and grew exponentially for 6 days thereafter, and the rate of growth decreased after day 15. The excretion values of 5H6MI2C and 5-S-CD in melanoma-bearing mice on day 15 were three orders of magnitude higher than those of control mice. The excretion value of 5H6MI2C started to increase as early as day 6 and increased rapidly in parallel with the tumour volume. For example, between days 9 and 12, the tumour volume increased 7-fold, while the 5H6MI2C value 9-fold. After day 15, the excretion value began to decrease, even though the tumour volume still increased. This may be related to the onset of tumour necrosis.

6H5MI2C was also consistently found in the melanotic urine. However, its excretion level was much lower than that of 5H6MI2C. For example, the average ratios of 5H6MI2C to 6H5MI2C were 5.8 and 10.7 for days 9 and 15, respectively.

The excretion value of 5-S-CD showed a pattern similar to that of 5H6MI2C; it increased 10-fold between days 9 and 12. However, some differences were noted: a temporary increase at day 3 and a slight increase even after day 15. The high value on day 3 may be ascribed to lysis of the melanoma cells inoculated and that after day 15 may also be related to lysis of the melanoma cells due to necrosis.

Of the two markers 5H6MI2C and 5-S-CD, the former appears to reflect better the

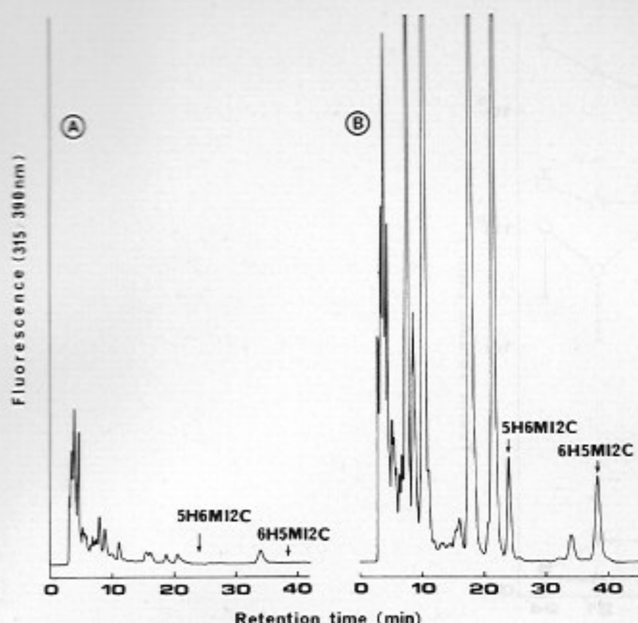

Fig. 1. HPLC chromatograms of urine samples from a mouse. (*A*), 3 days before tumour inoculation; (*B*), 15 days after B16 melanoma inoculation. For comparison, a 1/100 aliquot of each 24-h urine sample was injected and analysed under the same detection conditions: gain, ×1; attenuation, 64. The excretion values of 5H6MI2C and 6H5MI2C in the sample B were 314 and 23 nmol/day, respectively.

progression of melanoma at the early stage: it had a lower control level and a higher excretion on day 9, when the tumour was barely detectable.

The excretion value of dopamine also increased with the tumour volume between days 9 and 18 (Table I). The dopa excretion in melanoma-bearing mice was low (approx. 0.6 nmol/day for days 18–24), but much higher than the control level (approx. 0.01 nmol/day).

Table I. *Urinary excretion of melanin-related metabolites in B16 melanoma-bearing mice (n=8)*

Days after inoculation	5H6MI2C (nmol/day)		5-S-CD (nmol/day)		Dopamine (nmol/day)		Tumour volume (mm³)[a]	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
−3	0.24	<0.14–0.40	0.35	0.20–0.40	2.8	1.3–4.6	–	–
3	0.16	<0.11–0.22	5.6	1.9–15	7.0	3.7–14	<4	–
6	0.49	<0.12–1.4	1.5	0.44–4.6	4.1	2.1–5.4	<4	–
9	14	5.3–30	6.9	2.8–13	25	10–50	68	<4–196
12	125	26–338	73	13–212	120	24–275	495	63–1 080
15	218	104–373	149	89–199	123	72–176	1 730	726–2 820
18	122	49–263	303	187–566	177	98–301	2 530	1 270–5 320
21[b]	68	15–145	300	146–430	166	72–261	3 910	2 600–7 140
24[c]	129	48–213	431	323–573	147	25–261	6 300	5 600–6 780

[a] Calculated by the formula: long axis × (short axis)^2×1/2. The detection limit is 4 mm^3.
[b] n=7. One died on day 20.
[c] n=4. Three died between days 22 and 24.

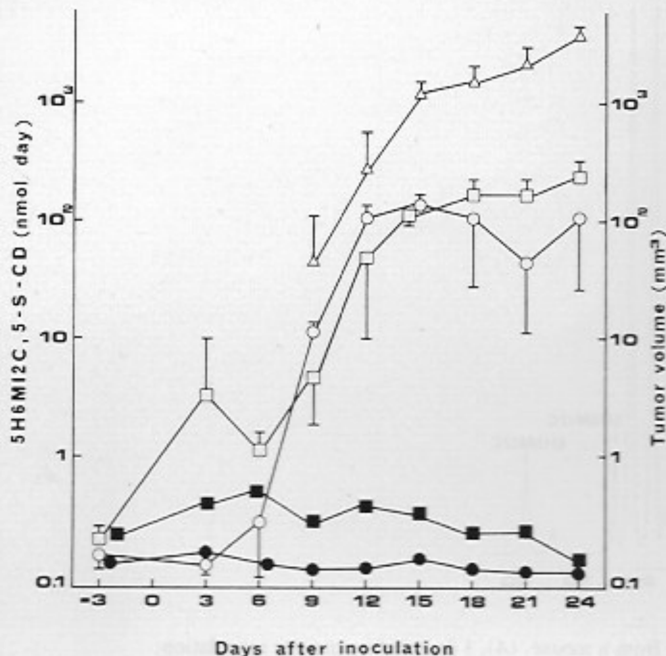

Fig. 2. Changes in the urinary excretion of 5H6MI2C and 5-S-CD and the tumour volume in B16 melanoma-bearing mice. Eight C57BL/6 mice were used for the experiment and 2 for control purposes. ○, 5H6MI2C in the experimental group; ●, 5H6MI2C in the control group; □, 5-S-CD in the experimental group; ■, 5-S-CD in the control group; △, tumour volume. Data for the experimental group were means ± SD, calculated for the log values.

DISCUSSION

Melanoma patients excrete a high level of 5-S-CD, while normal subjects excrete a low level (2), but the difference between these levels is rather small. This may be ascribed partly to the tyrosinase-independent production of 5-S-CD occurring outside of melanocytes (13, 14). In contrast to 5-S-CD, 5H6MI2C appears to be a specific metabolite for melanocytes. The urinary excretion of 5H6MI2C or its isomer 6H5MI2C reflects well the melanin formation in normal skin pigmentary system of humans (5, 15) as well as of mice (16).

The present study has demonstrated that urinary 5H6MI2C is a better marker of melanoma progression at the early stage in a model system of B16 mouse melanoma, whereas 5-S-CD appears to better reflect the lysis of melanoma cells. The analytical method employed is simple and reproducible, inasmuch as the urine sample is directly injected into the HPLC system without any pre-treatment.

Pavel et al. (4) have suggested, on the basis of a study with a limited number of patients, that among the eumelanin-related metabolites, 5H6MI is the most sensitive marker for follow-up of melanoma patients. However, we did not analyse this indole, for the following reasons. Firstly, 5H6MI is excreted mostly as conjugates with glucuronic or sulfuric acid and thus the tedious step of hydrolysis is required. Secondly, in contrast to 5H6MI2C, 5H6MI is extremely unstable and thus the standard solution must be prepared on every occasion of analysis. Thirdly, their recent work has indicated that 5H6MI may be formed also outside of melanocytes or under pathologic conditions (5).

REFERENCES

1. Prota G. Recent advances in the chemistry of melanogenesis in mammals. J Invest Dermatol 1980; 75: 122–127.
2. Agrup G, Agrup P, Andersson T, Hafström L, Hansson C, Jacobson S, Jönsson P-E, Rorsman H, Rosengren A-M, Rosengren E. 5 years' experience of 5-S-cysteinyldopa in malignant melanoma. Acta Derm Venereol (Stockh) 1979; 59: 381–388.
3. Duchon J, Matous B, Pavel S. Melanogenuria. In: Seiji M, ed. Phenotypic expression in pigment cells. Pigment cell 1981. Tokyo: Univ of Tokyo Press, 1981; 609–615.
4. Pavel S, Elzinga H, Muskiet FAJ, Smit JM, Mulder NH, Schraffordt Koops H. Eumelanin-related indolic compounds in the urine of treated melanoma patients. J Clin Chem Clin Biochem 1986; 24: 167–173.
5. Westerhof W, Pavel S, Kammeyer A, Beusenberg FD, Cormane R. Melanin-related metabolites as markers of the skin pigmentary system. J Invest Dermatol 1987; 89: 78–82.
6. Voorhess ML. Urinary excretion of dopa and metabolites by patients with melanoma. Cancer 1970; 26: 146–149.
7. Ito S, Kato T, Maruta K, Jimbow K, Fujita K. "Total" acidic metabolites of catecholamines in urine as determined by hydrolysis with hydriodic acid and liquid chromatography: application to patients with neuroblastoma and melanoma. Clin Chem 1985; 31: 1185–1188.
8. Rorsman H, Agrup G, Hansson C, Rosengren E. Biochemical recorders of malignant melanoma. In: MacKie RM, ed. Pigment cell, vol. 6. Basel: Karger, 1983; 93–115.
9. Ito S, Inoue S, Yamamoto Y, Fujita K. Synthesis and antitumor activity of cysteinyl-3,4-dihydroxyphenylalanines and related compounds. J Med Chem 1981; 24: 673–677.
10. Wakamatsu K, Ito S. Preparation of eumelanin-related metabolites 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, and their O-methyl derivatives. Anal Biochem [in press].
11. Ito S, Kato T, Maruta K, Fujita K, Kurahashi T. Determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in plasma, urine, and tissue samples by high-performance liquid chromatography with electrochemical detection. J Chromatogr 1984; 311: 154–159.
12. Pavel S, Slik WV. Analysis of eumelanin-related indolic compounds in urine by high-performance liquid chromatography with fluorimetric detection. J Chromatogr 1986; 375: 392–398.
13. Ito S, Fujita K. New possible routes for the biosynthesis of cysteinyldopas and related metabolites. In: Seiji M, ed. Phenotypic expression in pigment cells. Pigment cell 1981. Tokyo: Univ of Tokyo Press, 1981; 85–91.
14. Nimmo JE, Hunter JAA, Percy-Robb IW, Jay B, Phillips CI, Tayler WOG. Plasma 5-S-cysteinyldopa concentrations in oculocutaneous albinism. Acta Derm Venereol (Stockh) 1985; 65: 169–171.
15. Wirestrand L-E, Hansson C, Rosengren E, Rorsman H. Melanocyte metabolites in the urine of people of different skin colour. Acta Derm Venereol (Stockh) 1985; 65: 345–347.
16. Ekelund MC, Carstam R, Hansson C, Rorsman H, Rosengren E. Urinary excretion of 5-S-cysteinyldopa and 6-hydroxy-5-methoxyindole-2-carboxylic acid: differences between pigmented and albino mice. Acta Derm Venereol (Stockh) 1985; 65: 437–439.