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Correlation between Urinary Melanin-related Metabolites and Tumour Weight in Melanoma-bearing Mice

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Melanoma tissues produce not only 5-S-cysteinyldopa (5-S-CD), a pheomelanin precursor, but also 5,6-dihydroxyindoles, eumelanin precursors. We compared 5-S-CD and eumelanin-related metabolites regarding the correlation of their production in melanoma and excretion in urine, versus the weight of B16 mouse melanoma. It was found that B16 melanoma tissues produce 5,6-dihydroxyindole-2-carboxylic acid and its two O-methyl derivatives, the latter of which are partly conjugated with sulphuric acid and then excreted. These indolic metabolites are referred to as total indoles. Both total indoles and 5-S-CD in melanoma and in urine correlated well with each other and reflected the tumour weight. Total indoles had a four-fold greater level of excretion than 5-S-CD, suggesting that the urinary excretion of total indoles is a quantitatively more significant marker of melanoma progression in this melanoma. ***Key words: 5(6)-Hydroxy-6(5)-methoxyindole-2-carboxylic acids; Sulphate conjugates; 5-S-Cysteinyldopa; Melanoma.***

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In melanocytes, the amino acid tyrosine is oxidised to dopaquinone by the action of tyrosinase. The addition of cysteine (or glutathione) to dopaquinone gives the pheomelanin precursor 5-S-cysteinyldopa (5-S-CD) along with minor isomers (1). 5-S-CD has been most extensively used as a biochemical marker of melanoma progression (2, 3). In the absence of SH compounds, however, the spontaneous reaction of dopaquinone leads to the formation of 5,6-dihydroxyindole (5,6DHI) and 5,6-dihydroxyindole-2-carboxylic acid (5,6DHI2C), the eumelanin precursors (4). These indoles are partly O-methylated in melanocytes, released to circulation, and conjugated to some extents with glucuronic or sulphuric acid in the liver (5). It has been known for some years that these eumelanin-related metabolites are also excreted copiously in melanoma patients (6, 7).

We have been carrying out the studies intended to determine which of the two markers, 5-S-CD and eumelanin-related metabolites, better reflects the progression of melanoma (8, 9). We have shown that in mice bearing B16 melanoma, 5-hydroxy-6-methoxyindole-2-carboxylic aid (5H6MI2C), a eumelanin-related metabolite, had a higher excretion level in the earlier stage of melanoma progression, while 5-S-CD had a higher excretion level in the later stage (8). In this study, using the same mouse model, we compared 5-S-CD and eumelanin-related metabolites regarding the correlation of their production in melanoma tissue and excretion in urine, versus the tumour weight.

MATERIALS AND METHODS

Chemicals

5-S-CD, 5H6MI2C and its isomer 6-hydroxy-5-methoxyindole-2-carboxylic acid (6H5MI2C) were prepared chemically as described by us (10, 11). Sulphates and glucuronates of 5H6MI2C and 6H5MI2C have been detected in urine of a melanoma patient with widespread metastasis (manuscript in preparation).

The sulphates 5H6MI2C-S and 6H5MI2C-S were prepared by treating the free indoles with sulphur trioxide trimethylamine complex (12). Thus, a mixture of 1.0 mg of 5H6MI2C and 11 mg of sulphur trioxide trimethylamine complex in 0.3 ml of pyridine was heated at 70°C. The reaction was monitored by HPLC and found to be complete (98%) in 2 h. Therefore, the reaction was stopped by diluting the mixture 1 000-fold with the mobile phase for HPLC. For the preparation of 6H5MI2C-S, the reaction proceeded much more slowly and the yield was 88% in 4 h. All other chemicals were of analytical grade and purchased either from Sigma Chemical Co. (St. Louis) or from Wako Pure Chemical Industries (Osaka).

Animal experiments

B16 melanomas were maintained by subcutaneous inoculation of tumour cells on the back of C57BL/6 mice. Suspension of melanoma cells were prepared by homogenisation of

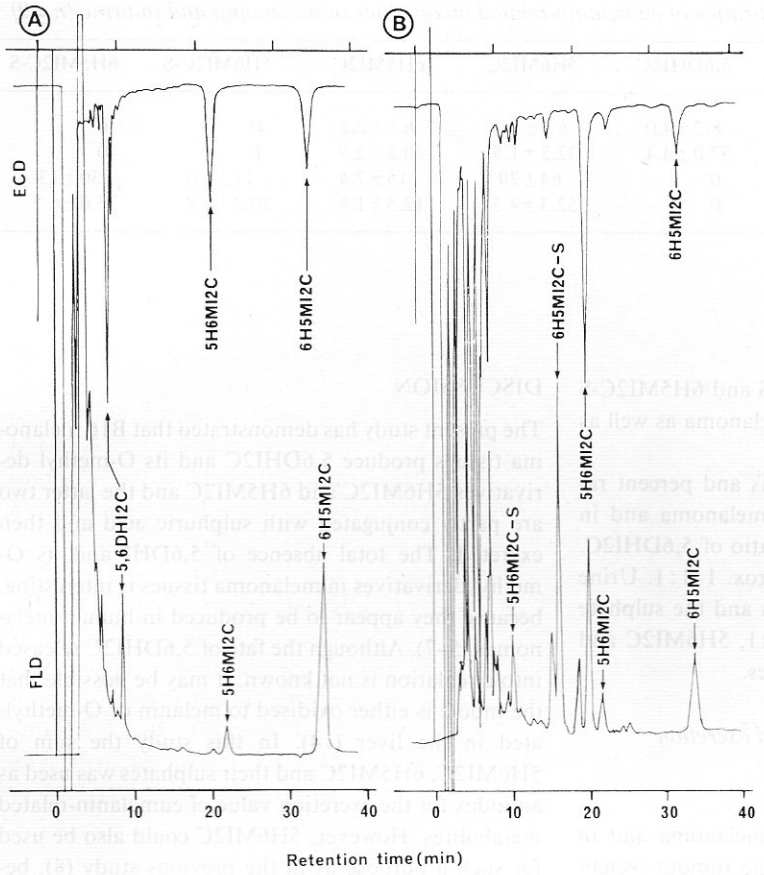

Fig. 1. HPLC chromatograms of melanoma and urine samples from a B16 melanoma-bearing mouse. (*A*) Melanoma extract; (*B*) 24-h urine sample. Electrochemical detection (ECD) was performed at +750 mV vs. an Ag/AgCl reference electrode, and fluorescence detection (FLD) was at 315 and 420 nm for excitation and emission, respectively (9).

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 in 0.5 ml was inoculated subcutaneously into axillary region of 12 male C57BL/6 mice (5 weeks old).

Animals were killed between days 18 and 28 and tumours were excised and weighed. One day before killing, urine samples were collected for 24 h in a beaker containing 1 ml of 20% acetic acid and 20 mg of sodium metabisulphite. Tumours weighing between 0.5 g and 4.0 g were used for analysis.

High-performance liquid chromatography (HPLC)

Indoles in urine and melanoma samples were determined by direct injection of urine samples or tumour extracts. Urine samples were centrifuged and 10-µl aliquots were injected into HPLC. Melanoma tissues were homogenised with 9 vol of 0.4 M $HClO_4$, centrifuged, and injected. Although organic sulphates are known to be unstable in acid, 5H6MI2C-S and 6H5MI2C-S were found to be stable enough in 0.4 M $HClO_4$: decomposition was less than 5% after 3 h at 25°C. The HPLC conditions were similar to those described by us (8, 9): an EICOM EC-100 electrochemical detector was used for the free indoles 5,6DHI2C, 5H6MI2C and 6H5MI2C; a JASCO 820-FP spectrofluorimeter was for the sulphates 5H6MI2C-S and 6H5MI2C-S.

5-S-CD in urine samples was determined after alumina extraction (13) and 5-S-CD in melanoma extracts by direct injection. The HPLC conditions were similar to those described by us (13); pH of the mobile phase was changed to 3.10.

RESULTS

HPLC analysis

HPLC analysis of melanoma extracts showed the presence of 5,6DHI2C, 5H6MI2C and 6H5MIC2 in a comparable ratio (Fig. 1 A). 5,6DHI and its O-methyl derivatives were not detected. On the other hand, urine samples contained the sulphate conjugates 5H6MI2C-S and 6H5MI2C-S in addition to free 5H6MI2C and 6H5MI2C (Fig. 1 B). Neither free 5,6DHI2C nor conjugates of 5H6MI2C and 6H5MI2C with glucuronic acid were detected in urine. From these results, the sum of 5,6DHI2C,

Table I. *Concentrations and percent ratios of eumelanin-related metabolites in melanoma and in urine (n=9)*

Sample	Unit[a]	5,6DHI2C	5H6MI2C	6H5MI2C	5H6MI2C-S	6H5MI2C-S
Melanoma	nmol/g tumour	8.2±4.0	6.9±2.3	6.6±2.2	0	0
	Percent ratio	37.0±4.4	32.2±1.9	30.8±2.9	0	0
Urine	nmol/day/g tumour	0	64±29	15±7.4	11±6.0	30±13
	Percent ratio	0	52.3±4.3	12.5±1.8	10.2±1.8	25.0±5.5

[a] Mean ± SD.

5H6MI2C, 6H5MI2C, 5H6MI2C-S and 6H5MI2C-S is referred to as total indoles in melanoma as well as in urine.

Table I represents concentrations and percent ratios of the individual indoles in melanoma and in urine. In melanoma extracts the ratio of 5,6DHI2C, 5H6MI2C and 6H5MI2C was approx. 1:1:1. Urine samples contained the free indoles and the sulphate conjugates in a ratio of approx. 2:1, 5H6MI2C and 6H5MI2C-S being the major indoles.

Correlation between production and excretion of melanin-related metabolites and tumour weight

The contents of total indoles in melanoma and in urine correlated fairly well with the tumour weight (Fig. 2A, B). A strong correlation was found between the production of 5-S-CD and the tumour weight (Fig. 2C). There was also a correlation between the excretion of 5-S-CD and the tumour weight (Fig. 2D).

Correlation was then examined between the contents of total indoles and 5-S-CD in melanoma and in urine. Good to excellent correlation was found between the contents of these two types of metabolites (Fig. 3). Although total indoles and 5-S-CD were found in melanoma tissues at the same levels, total indoles had a four-fold greater level of excretion than 5-S-CD (Table II).

DISCUSSION

The present study has demonstrated that B16 melanoma tissues produce 5,6DHI2C and its O-methyl derivatives 5H6MI2C and 6H5MI2C and the latter two are partly conjugated with sulphuric acid and then excreted. The total absence of 5,6DHI and its O-methyl derivatives in melanoma tissues is interesting, because they appear to be produced in human melanomas (5–7). Although the fate of 5,6DHI2C released into circulation is not known, it may be possible that the indole is either oxidised to melanin or O-methylated in the liver (14). In this study the sum of 5H6MI2C, 6H5MI2C and their sulphates was used as an index for the excretion value of eumelanin-related metabolites. However, 5H6MI2C could also be used for such a purpose as in the previous study (8), because it constitutes a half of total indoles in urine and its percent ratios are markedly constant (Table I).

Both total indoles and 5-S-CD in urine correlate well with each other and reflect the tumour weight. In this regard, it should be mentioned that 5-S-CD level in plasma has recently been shown to correlate with the size of tumour masses in mice (15). Hansson (16) has also found that the urinary excretion of 6H5MI2C correlates well with that of 5-S-CD during PUVA treatment but not under normal conditions.

There are some discrepancies between the present results and those in our previous report (8): in the

Table II. *Concentrations and ratios of total indoles and 5-S-CD in melanoma and in urine (n=9)*

Sample	Concentration[a]	Total indoles	5-S-CD	Total indoles/ 5-S-CD ratio
Melanoma	nmol/g tumour	22±8	20±4	1.05±0.22
Urine	nmol/day/g tumour	121±49	28±12	4.44±0.98

[a] Mean ± SD.

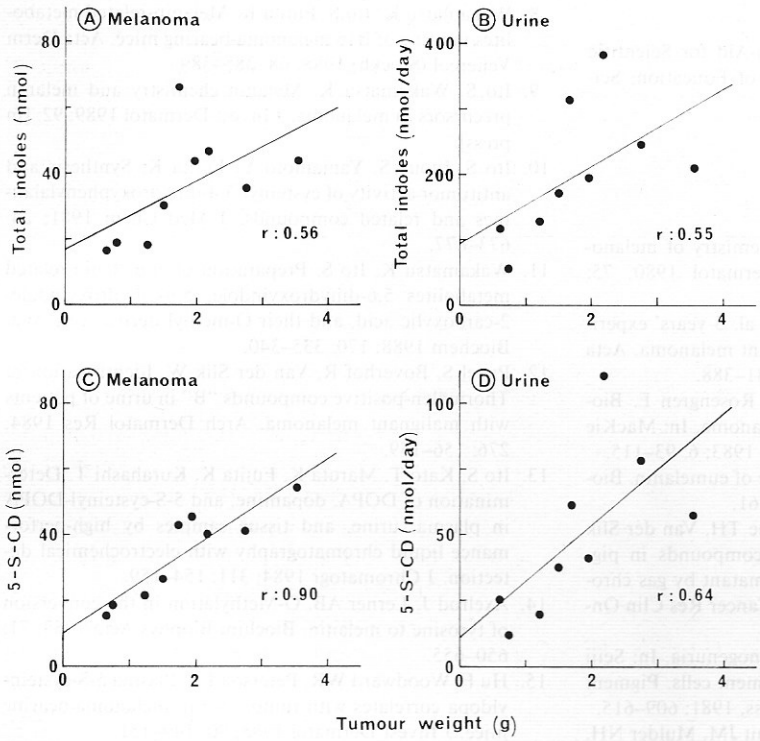

Fig. 2. Correlation between the tumour weight and the contents of melanin-related metabolites. Correlation with the contents of (*A*) total indoles in melanoma; total indoles in urine (*B*); 5-S-CD in melanoma (*C*); 5-S-CD in urine (*D*).

previous paper, the ratio between urinary 5H6MI2C and 6H5MI2C was approx. twice as high and the excretion values of 5H6MI2C and 5-S-CD were approx. 2 and 3 times as high, respectively. These discrepancies may be ascribed to the one-year difference in the time of animal experiments as well as to the difference in the methods to measure tumour mass.

Finally, it should be noted that total indoles had a four-fold greater level of excretion than 5-S-CD, while these two markers showed no difference in production (Fig. 3). Several explanations may account for this phenomenon: 1) a higher rate of oxidation of 5-S-CD during circulation, 2) a higher rate of release of indoles from melanoma cells, and 3) a higher rate of clearance of total indoles. Whatever the reason is, the higher excretion of total indoles makes it a quantitatively more significant marker of melanoma progression in B16 melanoma-bearing mice.

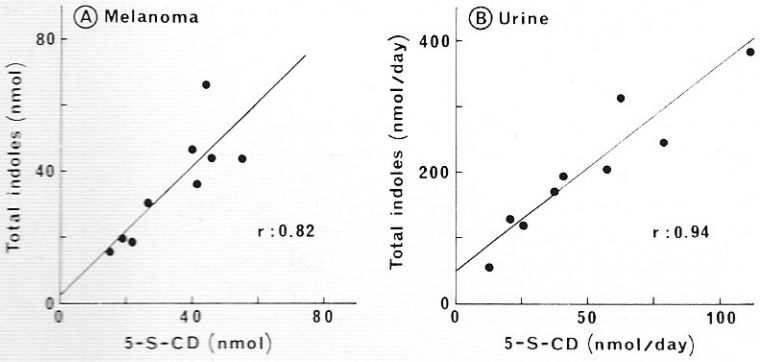

Fig. 3. Correlation between the contents of 5-S-CD and total indoles in melanoma (A) and in urine (B).

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