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Tyrosinase Activity in Serum from Patients with Malignant Melanoma*

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A preparation procedure is presented for the determination of tyrosinase-catalysed stereo-specific dopa oxidase activity in serum. Purification is obtained by separation on a Phenyl–Sepharose hydrophobic interaction column, followed by Con-A–Sepharose chromatography. Five out of seven sera from patients with widespread melanoma metastases were found to contain detectable quantities of tyrosinase. There was no tyrosinase activity in seven sera from patients with other malignancies, nor in six other control sera from individuals without malignancies. One serum which showed high tyrosinase activity was processed as above and studied by SDS–PAGE. A dopa-reactive band with an apparent MW of 66 kD was present in the gel, i.e. at the same place as that of the soluble tyrosinase of cultured human malignant melanoma cells. The protein was found to have the same pI at isoelectric focusing, and eluted in the same way from the preparation columns used, as did soluble tyrosinase. ***Key words: Cysteinyldopa; Dopa; Tumour marker.***

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With the advancement of cancer therapy, markers for the early detection of malignancy have become of increasing importance. There are only a few tumours where we have a chemical marker for metastases, widespread malignant melanoma being one of them.

It has long been known that the urine of patients with advanced melanoma darkens on standing. The reason for this is the presence of large amounts of indolic melanin precursors, as described by Thormählen (1). A procedure described by him for the determination of these compounds in urine was for a long time widely used as a marker for melanoma metastases. For many years Duchon and his collaborators have extensively studied and characterized the indolic substances in urine from melanoma patients (2). In their experience these analyses have been shown to monitor the progress of the disease.

The presence in urine of another group of substances, dopa and dopa-derivatives has also been used to screen for melanoma metastases. The compounds are however produced in large quantities in the nervous system and therefore the contribution of these compounds from melanoma is easily obscured by the normal variations (3).

The important melanin precursor, 5-S-cysteinyldopa, has been demonstrated in normal urine and increased concentrations have been observed in the urine of patients with malignant melanoma (4). Studies have shown that determination of urinary cysteinyldopa can detect a metastatic mass of about 1 g (5). Initially 5-S-cysteinyldopa was determined by fluorometry (6), later by HPLC and electrochemical detection (7). The way the excretion of this amino acid in urine varies with the season, medication, UV-light exposure, and hormonal influence has been studied (8–11).

A substance that is excreted mainly from malignant melanocytes should be a clinically useful marker. Tyrosinase, the enzyme catalysing the initial steps in melanin synthesis and present exclusively in melanocytes, could well be such a substance.

In 1969 Sohn et al. reported tyrosinase activity in serum from a patient with melanosis due to disseminated melanoma (12). Del Vecchio & Burnett studied melanoma patients and isolated a serum protein, which on immunoelectrophoresis showed some antigenicity in common with mouse tyrosinase (13). Nishioka et al., using the Pomerantz method for assay (14), found tyrosinase in serum from melanoma patients, but not in serum from patients with other malignancies (15). In contrast, Chen & Chavin, determining tyrosinase activity by detecting the formation of melanin from labelled dopa, found tyrosinase ac-

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Table I. *Sex, age, and clinical data of patients*

M = man, W = woman

W/65	Spring 1986, melanoma Clark III, 1.4 mm, with axillary node metastases. Possible liver metastases. Treatment: Interferon and cimetidine.
W/66	1983, melanoma Clark III–IV on back. 1986, metastases to hip region, inguinal nodes excised. Treatment: Vindesine, DTIC.
M/62	Spring 1986, melanoma Clark IV, 5 mm, on back. Later axillary, cutaneous, epipharyngeal and adrenal metastases. Treatment: Interferon, cimetidine.
W/64	Aug. 1976, melanoma Clark III, lower leg. 1984, inguinal metastases. 1987, pulmonary metastasis. Treatment: Interferon, cimetidine, later DTIC, vindesine, cisplatin.
M/64	1963, melanoma shoulder. Spring 1987, mental symptoms (tumour in right cerebral hemisphere). Autumn 1987, cutaneous melanoma metastases. Treatment: Interferon, cimetidine.
W/57	1976, melanoma thigh. 1980, 1983, local relapse. 1984, relapse. Since 1986, several metastases excised, the latest in May 1988. Treatment: Interferon, ranitidine.
M/70	Spring 1985, melanoma Clark IV, 3.7 mm on back. Autumn 1986, axillary node metastasis. Autumn 1987, growing metastases. Treatment: Interferon.
W/70	Spring 1985, melanoma Clark III, 0.95 mm on back. Spring 1987, metastases in liver from malignant tumour with low differentiation. Death in Oct 1987. At autopsy, gastric cancer with widespread metastases, no melanoma.
W/67	Jan. 1987, pancreatic cancer with metastases to lymph nodes and spine.
M/62	Spring 1986, ascites, primary hepatic cancer.
M/72	Dec. 1986, prostatic cancer with metastases to skeleton.
W/51	Jan. 1986, rectal cancer, progressive.
W/51	Spring 1984, mammary cancer with progressing metastases.
M/66	Spring 1987, small-cell bronchial cancer with progress.
W/45	1985, non-Hodgkin lymphoma in abdominal and pelvic nodes.

tivity in serum not only from melanomas, but also from other malignancies (16). Vachtenheim et al. recently published a study on the detection of tyrosinase in serum from melanoma-bearing hamsters, using a radio-immunoassay. Using rabbit polyclonal tyrosinase antibodies, they could also detect tyrosinase protein in control hamsters, but failed to show tyrosinase in melanoma-bearing mice or men (17).

The assay of tyrosinase activity in serum is fraught with several sources of error. The non-enzymatic oxidation of dopa by, for instance hemoproteins (18), must be considered when evaluating tyrosinase activity, as this non-specific oxidation is quantitatively important compared with the levels of tyrosinase activity that can be present in serum, even in patients with a large tumour mass.

We describe here a rather simple purification of serum, which allows the detection of pathologically increased stereospecific dopa oxidase activity in certain melanoma patients, using a method where the non-specific oxidation is taken into account (19).

MATERIALS AND METHODS

Sera from 8 patients with melanoma and from 7 patients with other malignancies were obtained from the Oncological Clinic at the General Hospital, Malmö, Sweden. Relevant data regarding the clinicopathological findings of the patients are given in Table I. Six sera from members of the staff or from patients with non-malignant, non-hyperpigmented dermatoses at the Dermatological Clinic, University Hospital, Lund, Sweden served as further controls. All sera were drawn in August–November 1987, except for one patient (Woman/64) from whom serum was collected in March 1988.

Cultured human melanoma metastasis cells (IGR 1) were a gift from Dr C. Aubert, Marseille, and have been kept in culture since 1982 at the Tornblad Institute, University of Lund, by methods described previously (20).

Sample preparation

Solid ammonium sulphate was mixed with crude serum to a final concentration of 1 M and the sample was centrifugated at 30000 g for 30 min. The supernatant was then passed through a small column (50×5.5 mm) containing Phenyl–Sepharose (Pharmacia, Sweden), equilibrated with 1 M $(NH_4)_2SO_4$, 20 mM KH_2PO_4, pH 7.0. After washing the column with 2×5 ml of 1 M ammonium sulphate, adjusted to pH 7.0, elution was performed with 10 ml 20 mM KH_2PO_4 pH 7.0. The eluate was then passed through a column (20×5.5 mm) containing Concanavalin-A–Sepharose (Pharmacia, Sweden) in 4 mM KH_2PO_4, 1 M KCl, pH 7.0. The column was washed with 5 ml 4 mM KH_2PO_4, 1 M KCl, pH 7.0 and 5 ml 4 mM KH_2PO_4 pH 7.0, then eluted with 0.5 M methyl-alfa-D-mannopyranoside, 4 mM KH_2PO_4, pH 7.0, in 1-ml fractions. The eluates were analysed for tyrosinase activity.

Tyrosine assay

The measurement of tyrosinase activity was based upon the determination of 5-S-L-cysteinyl-L-dopa formed in the presence of D,L-dopa and L-cysteine. 0.3 ml of the eluate from Concanavalin-A (ConA) was added to 0.7 ml of a solution, 1 mM L-dopa, 1 mM D-dopa and 3 mM L-cysteine in 0.5 M of phosphate buffer, pH 7.4. The mixture was incubated at 37°C for 2 min under gentle air bubbling. 0.1 ml of the incubate was then added to 0.9 ml of 0.4 M perchloric acid, and after centrifugation, the content of 5-S-cysteinyldopa in the supernatant was determined by means of HPLC and electrochemical detection (21).

The HPLC-system consisted of a LKB 22150 HPLC pump, a sampling-valve injector Rheodyne model 7120 (Rheodyne, Berkley, Calif., USA), equipped with a 100 µm loop; the

Table II. *Tyrosinase activity in serum from melanoma patients and controls*

Numbers in parentheses. Tyrosinase activity given as nmole 5-S-L-cysteinyl-L-dopa formed/min and per ml serum

Diagnosis	Tyrosinase activity (nmole)
Melanoma metastases ($n=17$)	0.65, 0.45, 0.18, 0.17, 0.16, 0.00, 0.00
Melanoma without metastases, widespread adenocarcinoma metastases ($n=1$)	0.00
Other malignancies ($n=7$)	0.00–0.00
Healthy or non-malignant skin disorders ($n=6$)	0.00–0.00

column 125×4.6 mm, used was packed by the upward slurry method with Nucleosil C_{18}, 5 µm (Machery, Nagel & Co., Duren, GFR); mobile phase 6 g methanesulphonic acid, and 3 g phosphoric acid per litre of MilliQ purified water; pH was adjusted to 3.0. Flow rate, 1.5 ml/h. An external standard containing a known amount of 5-S-L-cysteinyl-L-dopa was injected immediately prior to each determination.

Detection. A model LC-10 amperometric detector (Bioanalytical Systems Inc., West Lafayette, Ind., USA) was used. The detector was operated at 0.75 V vs. an Ag/AgCl (3 M NaCl) reference electrode. The working electrode was prepared from carbon paste, CPO (Bioanalytical Systems Inc.). Chromatography integrator: SP 4270 (Scandia Metric AB, Solna, Sweden).

SDS–PAGE. In one case the tyrosinase protein found in the serum was further characterized. Three eluates from the column containing ConA were concentrated with Centricon Microconcentrators (Amicon, Danvers, Mass, USA). MW determination was performed with SDS–PAGE slab gel (1.5 mm thick) electrophoresis according to Laemmli, using a Bio-Rad Electrophoresis apparatus (Bio-Rad, Richmond, Calif., USA). Sample preparation: 50 µl of the sample was mixed with 50 µl of solubilization solution containing 20% glycerol, 4% SDS, 0.125 M Tris-HCl, pH 6.8, and small amounts of bromophenolblue. No thiol reagents were added and no boiling was done, in order to preserve the activity of the enzyme. As a MW marker for the Coomassie Brilliant Blue staining, we used the Pharmacia Electrophoresis Calibration Kit for low molecular weight proteins (Pharmacia, Sweden).

Running buffer (upper and lower): 8.3 mM Tris (1/3 of concentration used by Laemmli) 0.192 M glycine, 0.1% SDS, pH 8.3.

Running conditions: 7 mA for 30 min, 25 mA for 5 1/2 h.

Staining: The gel was split longitudinally into two, one part stained with Coomassie Brilliant Blue 0.1%, and the other by enzymatic staining using 0.08 g L-dopa and 0.05 g L-tyrosine in 200 ml 0.1 M bisTris, pH 6.5.

Isoelectric focusing

Isoelectric point determination of the protein with tyrosinase activity was performed on similar extracts using flat-bed IEF. The casting was made using the Pharmacia capillary casting mould (Pharmacia). For creating the pH-gradient, Pharmalyte pH 3-10 (Pharmacia) was used.

The IEF gels were 230×115×1.0 mm, and for sample application we used filter papers size 20×15 mm. 5% acrylamide-bis was used for cross-linking.

Running conditions: Prefocusing for 30 min, 150 mA at a fixed effect of 10 W. Focusing for 90 min, 150 mA at a fixed effect of 30 W. The tyrosinase band in the gel was visualized by enzymatic staining with 200 mg L-dopa, in 200 ml 0.1 M Tris, pH 7.5.

RESULTS

The study showed the presence of tyrosinase activity, measured as stereospecific dopa oxidase activity in the sera of 5 of 7 patients with widespread malignant melanoma. There was no tyrosinase activity in the sera from patients with other malignancies, nor in the other control sera.

We were unable to detect any stereospecific dopa oxidase activity in the sera of 2 of our patients with malignant melanoma. One of the patients (W/57) had amelanotic melanoma cells in her tumour. She had recurrent cutaneous metastases, but was otherwise in good health. In the other patient (M/70) some of the cells in the primary melanoma were strongly pigmented; the metastases have not been examined microscopically.

The patient (W/70) with melanoma excised in 1985, and who later developed gastric cancer, was examined in spring 1987 with a liver scan because of pathologic serum transaminase values. The scan suggested metastases. Cytologic examination showed metastasis from a tumour of low differentiation and of unknown origin. She died in Oct 1987. At post-mortem examination, a gastric cancer with infiltration into the large bowel was found, with metastases to the liver and with peritoneal carcinomatosis. However, no signs of malignant melanoma metastases were found.

The serum with the highest tyrosinase activity was further analysed. SDS–PAGE showed a faint but clearly visible dopa-stained band with the apparent MW 66 kD, which corresponds to that of the 'soluble' tyrosinase obtained from human melanoma cells. Isoelectric focusing of the sample also revealed a band stained with dopa with the same pI, 4.55, as that of the purified soluble tyrosinase. The protein also eluted in the same way as the soluble tyrosinase on a

phenyl–Sepharose hydrophobic interaction column (22).

DISCUSSION

The stereospecific dopa oxidase activity found in serum corresponds to a protein with many characteristics of soluble tyrosinase isolated from cultured human malignant melanoma cells (IGR1).

In our culture of human melanoma cells, we have found a specific activity of 40 nmole dopa oxidized/min per mg protein. With a protein content around 10%, this would mean an activity of 4000 nmole/min and g cell, wet weight. The level of dopa oxidase activity found, 0.3 nmole/min/ml/sera, corresponds roughly to an activity of 900 nmole/min present at one time in the blood volume. This amount of tyrosinase may be derived from necrosis of melanoma tissue, corresponding to 0.2 g lysed melanoma cells, but may also be due to a more biologic release from a larger quantity of melanoma tissue. The high content of sialic acid of tyrosinase may prevent the trapping of the enzyme by the mannose receptors present in liver, with resulting diminished rate of removal from the plasma.

Untreated serum has a non-specific dopa oxidation too high for tyrosinase activity to be discernible. Adsorption of tyrosinase to ConA–Sepharose and subsequent elution eliminates to a large extent this non-specific oxidation, but the non-specific oxidation is still too great to allow a quantification of tyrosinase activity. This oxidation is probably due to the presence of hemoproteins. When passing the serum sample through a phenyl–Sepharose HIC in the presence of ammonium sulphate, hemoproteins elute earlier than the protein with tyrosinase activity. Passing the serum through a phenyl–Sepharose HIC alone, however, does not diminish non-specific dopa oxidation sufficiently to allow detection of tyrosinase activity.

Under our experimental conditions the detection limit for dopa oxidase activity was 0.01 nmole 5-L-cysteinyl-L-dopa formed/min/ml serum. The absence of detectable tyrosinase activity in serum from patients with other malignancies and from other controls may of course be due to levels of tyrosinase that can be detected only with an assay of greater sensitivity.

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REFERENCES

1. Thormählen J. Mitteilungen über einen noch nicht bekannten Körper im patologischen Menschenharn. Virchows Arch Pathol Anat 1887; 108: 317–322.
2. Matous B, Mechl Z, Sopkova B, Duchon J, Pavel S, Budesinska A, Kocent A. The excretion of Thormählen positive melanogens in melanoma patients and its clinical significance. Eur J Cancer Clin Oncol 1980; 16: 383–388.
3. 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.
4. Agrup G, Agrup P, Andersson T, Hafström L, Jacobsson S, Jönsson PE. 5 years experience of 5-S-cysteinyldopa in melanoma diagnosis. Acta Derm Venereol (Stockh) 1979; 59: 381–388.
5. Rosdahl I, Rorsman H. An estimate of the melanocyte mass in humans. J Invest Dermatol 1983; 81: 278–281.
6. Rorsman H, Rosengren AM, Rosengren E. Fluorometry of a dopa peptide and dopa thioethers. In: McGovern V, Russel P, eds. Pigment Cell, vol. 1. Basel: Karger, 1973; 171–179.
7. Hansson C, Agrup G, Rorsman H, Rosengren AM, Rosengren E. Analysis of cysteinyldopas, dopa, dopamine, noradrenaline and adrenaline in serum and urine using High Performance Liquid Chromatography and electrochemical detection. J Chromatogr 1979; 162: 7–22.
8. Agrup G, Falck B, Rosengren AM, Rorsman H, Rosengren E. Exposure to sunlight and urinary excretion of 5-S-cysteinyldopa. In: Riley V, ed. Pigment Cell, vol. 2. Basel: Karger, 1976; 284–289.
9. Stewart RM, Miller S, Gunder M. Urinary 5-S-cysteinyldopa in Parkinsonism after dopa and carbidopa. Acta Derm Venereol (Stockh) 1983; 63: 97–101.
10. Hansson C, Wirestrand LE, Aronson A, Rorsman H, Rosengren E. Urinary excretion of 6-hydroxy-5-methoxyindole-2-carboxylic acid and 5-S-cysteinyldopa during PUVA treatment. Photodermatology 1985; 2: 52–57.
11. Carstam R, Hansson C, Rorsman H, Rosengren E, Sjöberg N-O, Wirestrand L-E. Urinary excretion of melanocytic metabolites in fertile women. Acta Derm Venereol (Stockh) 1985; 65: 543–545.
12. Sohn N, Gang H, Gumport SL, Goldstein M, Deppisch LM. Generalised melanosis secondary to malignant melanoma. Report of a case with serum and tissue tyrosinase studies. Cancer 1969; 24: 897–903.
13. Del Vecchio V, Burnett J. Immunochemical detection of tyrosinase in serum of patients with metastatic malignant melanomas and from Hardy-Passey mouse melanomas. Aust J Dermatol 1976; 17: 26–29.
14. Pomerantz SH. Tyrosine hydroxylation catalyzed by hu-

man tyrosinase: an improved method of assay. Biochem Biophys Res Commun 1964; 16: 188.
15. Nishioka K, Romsdahl MM, McMurtrey MJ. Adaptation of tritiated tyrosine assay to serum tyrosinase and its specific elevation in melanoma. In: Klaus SN, ed. Pigment Cell, vol. 5. Basel: Karger, 1979: 300–304.
16. Chen YM, Chavin W. Serum tyrosinase and tyrosinase-affecting factors in human malignant disease. In: Klaus SN, ed. Pigment Cell, vol. 5. Basel: Karger, 1979: 293–299.
17. Vachtenheim J, Duchon J, Matous B. Tyrosinase of hamster melanoma: its purification and estimation by radioimmunoassay. Comp Biochem Physiol 1987; 87B(4): 709–714.
18. Agrup G, Hansson C, Rorsman H, Rosengren E, Tegner E. Methaemoglobin-catalysed formation of dopa and 6-OH-dopa from tyrosine. Acta Derm Venereol (Stockh) 1983; 63: 152–155.
19. Jergil B, Lindbladh Ch, Rorsman H, Rosengren E. Dopa oxidation and tyrosine oxygenation by human melanoma tyrosinase. Acta Derm Venereol (Stockh) 1983; 63: 468–475.
20. Aubert C, Rouge F, Galindo JR. Tumorigenicity of human malignant melanocytes in nude mice in relation to their differentiation in vitro. J Natl Cancer Institute 1980; 64: 1029.
21. Agrup G, Edholm LE, Rorsman H, Rosengren E. Diastereomers of 5-S-cysteinyldopa. Acta Derm Venereol (Stockh) 1983; 63: 59–61.
22. Carstam R, Edner C, Hansson C, Lindbladh C, Rorsman H, Rosengren E. Metabolism of 5-S-glutathionyldopa. Acta Derm Venereol (Stockh) 1986; 66: Suppl. 126.