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Seasonal Variation of Tyrosinase Activity in Serum

BJÖRN SONESSON[1], SIV EIDE[2], HANS RORSMAN[1] and EVALD ROSENGREN[2]

Departments of [1]Dermatology and [2]Pharmacology, University of Lund, Sweden

Serum samples for the analysis of tyrosinase activity were obtained from 10 healthy subjects in autumn, winter and summer. Tyrosinase was purified from 100 μl serum by adsorption to concanavalin A sepharose, the tyrosinase adsorbed to the gel being separated from other components by centrifugation. The gel was suspended in a buffer containing 5-hydroxy-indole-3-acetic acid as an antioxidant and incubated for 2 min with L-cysteine and D-L-dopa at 37°C. The 5-S-L-cysteinyl-L-dopa formed was measured by HPLC and electrochemical detection. Tyrosinase has high stereo-specificity for the L-enantiomer of dopa, and correction for non-specific oxidation was made by simultaneous measurement of 5-S-L-cysteinyl-D-dopa formed from D-dopa. Whereas the oxidation of L-dopa catalysed by tyrosinase was inhibited by L-tyrosine, the non-specific oxidation of D-dopa was not. Mean serum tyrosinase activity was 0.9 nkatal/l in summer, 0.8 nkatal/l in autumn and 0.4 nkatal/l in winter. The range of tyrosinase activity was much higher in summer and autumn than in winter. ***Key words: cysteinyldopa; dopa; tyrosine.***

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H. Rorsman, Department of Dermatology, Lasarettet, S-221 85 Lund, Sweden.

The incidence of melanoma has steadily increased in Caucasian populations throughout the world during recent decades. Therefore the need of markers for use in early detection of metastasis and guidance of therapy has become increasingly urgent. The serum concentration of tyrosinase (EC 1.14.18.1), an enzyme exclusively synthesized in melanin-producing cells and responsible for the initial steps of melanin synthesis, might be such a clinically useful marker.

In 1969 Sohn et al. (1) reported tyrosinase activity to be present in serum from a patient with generalised melanosis secondary to disseminated melanoma. Del Vecchio & Burnett (2) subsequently isolated a serum protein from the blood of melanoma patients, which on immuno-electrophoresis manifested some antigenicity in common with mouse tyrosinase. Both Nishioka et al. (3) and Agrup et al. (4) found tyrosinase activity in the serum samples of melanoma patients with advanced metastatic disease but not in those of healthy controls or of patients with other malignancies. In 1987, Vachtenheim et al. (5), using a radioimmunoassay, showed tyrosinase to be present in serum from melanoma-bearing and control hamsters. A sensitive radioimmunoassay developed by Wittbjer et al. (6), with a detection limit of about 5 pmol/l, detected no tyrosinase in the sera of subjects recently unexposed to sunlight.

In 1983, Jergil et al. (7) reported the presence of tyrosinase activity in the media of human melanoma cell cultures, and Karg et al. (8) subsequently showed the tyrosinase in the culture media to be released by living melanoma cells and not from cell lysis.

For the past decade, we have extensively used the concentration of 5-S-L-cysteinyl-L-dopa formed at oxidation of dopa in the presence of cysteine, as a measure of tyrosinase activity. With D-dopa also present in the incubate, the quantity of 5-S-L-cysteinyl-D-dopa formed is an internal control of non-specific oxidation, since tyrosinase has high stereo-specificity for the L-enantiomer of dopa. For the measurement of tyrosinase activity in human serum, it became necessary to minimise the non-specific oxidation that may occur in the presence of proteins containing transition metals (9). This was effected by purification of the enzyme from serum by affinity chromatography (10). In the present study, a modification of this method was used to measure tyrosinase activity in serum samples from healthy subjects in autumn, winter and summer.

MATERIAL AND METHODS

Chemicals

5-hydroxyindole-3-acetic acid, L-cysteine, reduced glutathione and EDTA were obtained from Sigma Chemical Co, St Louis, USA. 5-S-L-cysteinyl-L-dopa was synthesized as described by Agrup et al. (11). All

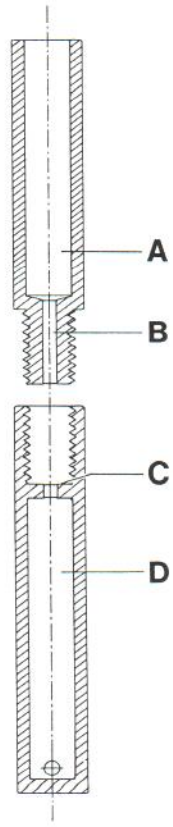

Fig. 1. Concanavalin A sepharose was introduced into chamber *A*. The small channel (*B*) was loaded with the gel by gentle centrifugation of the device. Filter paper (*C*) prevents the gel from reaching the waste chamber (*D*).

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HPLC reagents were of analytical grade, and all other chemicals of high commercial grades.

Subjects

The study was carried out in the southern Swedish city of Lund (lat. 55°–43'N, long. 13°12'E), where seasonal variation in available sunlight is marked. Blood samples were collected by cubital venepuncture from 10 healthy male subjects in autumn (September, October) 1992, winter (February, March) 1993 and summer (June) 1993. An unusually sunny summer with 949 h of sunshine during the months May to July 1992 preceded our investigation, though August was rather dull (12). At least 4 weeks had elapsed since the 1992 summer holiday before the autumn sera were taken and analysed. The spring of 1993 (April, May) was unusually sunny. As all the subjects had regular jobs, exposure to sunlight was mainly limited to weekends and summer vacations.

Sun-reaction skin types

A questionnaire concerning sunburn and suntanning history was used for the classification of sun-reactive skin types (13). Types III and IV were found in our group of healthy subjects.

Blood samples

Blood was obtained by venepuncture. The blood sample was kept at room temperature for 30 min and then centrifuged (3,000 g) for 10 min at 20°C, the serum obtained being analysed immediately.

Purification of tyrosinase from serum

Tyrosinase, a high-mannose glycoprotein, was purified by adsorption to Con-A (Pharmacia LKB, Uppsala, Sweden). With the use of the device illustrated in Fig. 1, 50 µl of Con-A was introduced into chamber A, the small channel B being loaded with the gel by gentle centrifugation of the device. After washing (by infiltration from chamber A by centrifugation into chamber D) the gel with 100 µl 4 mM KH_2PO_4, 1 M KCl, pH 7.2, and 100 µl 4 mM KH_2PO_4, pH 7.2, 100 µl of the serum to be analysed was placed in chamber A and the liquid removed by centrifugation of the device. The gel was washed with 50 µl 4 mM KH_2PO_4, 1 M KCl, pH 7.2 and 50 µl 4 mM KH_2PO_4, pH 7.2, the chamber A (including channel B) was unscrewed and the gel removed with 100 µl 0.1 M KH_2PO_4, pH 7.4, into a tube used for incubation. The activity of tyrosinase adsorbed to the gel was then analysed.

Tyrosinase assay

The Con-A gel suspension was incubated with 70 µl 0.1 M KH_2PO_4, pH 7.4, 10 µl 5 mM 5-hydroxyindole-3-acetic acid in 0.1 M KH_2PO_4, pH 7.4, 10 µl 33 mM L-cysteine and 20 µl 10 mM D,L-dopa at 37°C for 2 min under gentle air bubbling. The addition to the incubate of 5-hydroxyindole-3-acetic acid, which has a lower oxidation potential than dopa, limits the non-specific oxidation (data not shown). Incubation was interrupted by the addition of 80 µl 30 mM reduced glutathione in 1 M sodium acetate, pH 4.7. The amounts of 5-S-L-cysteinyl-L-dopa and 5-S-L-cysteinyl-D-dopa were determined by HPLC and electrochemical detection (11).

HPLC

To avoid system overload, from the high concentrations of dopa, a two-column chromatographic system was used for detection of 5-S-cysteinyldopas. In this system, column I (see below) separated dopa from 5-S-cysteinyldopas.

The HPLC system consisted of an LKB 22150 HPLC pump, flow rate 1.2 ml/min, a sampling-valve injector (Rheodyne model 7125, Rheodyne, Berkley, Calif., USA), a small boronate-column (*column I*: guard-column design, bed dimensions 20 × 2 mm, Alltech Ass., Deerfield Il., USA; packed with phenyl-boronic acid, 10 µm, Skandinaviska Gene-Tec AB, Kungsbacka, Sweden) used in line with a C 18 analytical column (*column II*: 150 × 4.6 mm Supelcosil LC-18-DB, 3 µm, Supelco. Inc., Bellafonte, PA, USA) and a mobile phase (18 mM H_3PO_4, 0.2 mM EDTA/L MilliQ purified water, pH adjusted to 2.5).

The analytical cell 5010 was used with an ESA Model 5200 Coulochem II detector (ESA, Bedford, MA, USA), set at +340 mV and −75 mV, and the detector was connected to an SP 4400 integrator (Spectra-Physics Analytical, Fremont, CA, USA).

The incubation was interrupted as described above, and 25 µl of the sample was applied to column I, previously equilibrated with 0.1 M sodium acetate buffer, pH 4.7 (4 × 250 µl). The column was then washed twice with 250 µl 0.1 M sodium acetate buffer, pH 4.7; at this pH most of the dopa present in the sample passed through the column, while 5-S-cysteinyldopas were retained. Then the injector valve was switched so that the mobile phase from the analytical column passed through column I and into column II. The 5-S-cysteinyldopas were eluted from column I and loaded on column II, which provided a clear separation of 5-S-L-cysteinyl-D-dopa and 5-S-L-cysteinyl-L-dopa. An external standard containing a known amount of 5-S-L-cysteinyl-L-dopa was injected immediately prior to every fourth measurement.

RESULTS

Validation of the method

A serum sample from a patient with disseminated melanoma which had a very high serum tyrosinase concentration was analysed as quadruplicates without purification. The same serum sample was also purified as quadruplicates, as described above, before measurement of tyrosinase activity. The four non-purified quadruplicates manifested stereospecific formation of 5-S-L-cysteinyl-L-dopa with a mean of 20.5 nkatal/l (C.V. = 10%). The mean value for identical sera purified in the same way as the sera of the healthy subjects above was 11.5 nkatal/l (C.V. = 10%). Thus the yield after purification was approximately 56%.

Variation of the method

In the autumn of 1992 the variation of the method was analysed by dividing serum samples from each of the 10 subjects into eight aliquots. The standard deviation in % of mean value varied from 9 to 26% in the 10 subjects (mean 17%).

Inhibition of dopa oxidation by tyrosine

Inhibition of dopa oxidation by 1 mM L-tyrosine resulted in values that were one third of those for the controls (Fig. 2).

Seasonal variation

The results reported from the autumn of 1992 were the mean of eight determinations (eight replica), while those from the winter and summer of 1993 were from single analyses. Tyrosinase activity above the detection limit of 0.1 nkatal/l serum, measured as stereospecific dopa oxidation, was found in sera from all subjects (autumn, winter and spring). The mean serum tyrosinase value was 0.8 nkatal/l (range 0.3–1.9 nkatal/l) in autumn, 0.4 nkatal/l (range 0.2–0.8 nkatal/l) in winter and 0.9 nkatal/l (range 0.4–2.0 nkatal/l) in summer. The values have not been corrected for recovery.

DISCUSSION

Under our experimental conditions the detection limit of serum tyrosinase activity was 0.1 nkatal/l. The present enzymatic method has a sensitivity approximately one order of magnitude higher than a RIA method previously reported (6). With the purification procedure described above, this new method permits relatively easy estimation of tyrosinase activity, measured as stereospecific dopa oxidase activity (10), in small amounts of

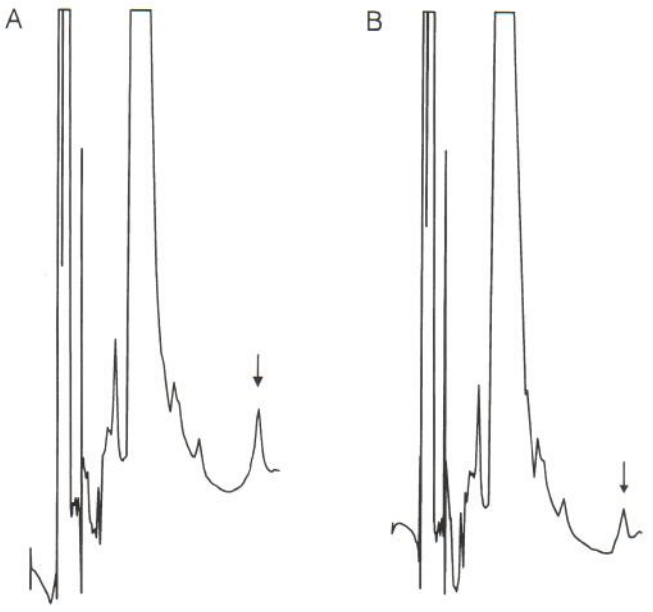

Fig. 2. Typical chromatogram showing inhibition of dopa oxidation by tyrosine (10). Serum preparation and tyrosinase assay were performed as described in the text. The incubation mixture for the assays either contained no tyrosine (A), or 1 mM L-tyrosine (B). The *arrows* indicate 5-S-L-cysteinyl-L-dopa peaks, which are immediately preceded by the smaller 5-S-L-cysteinyl-D-dopa peaks.

human serum. The device designed for this serum purification procedure enabled tyrosinase activity to be measured in 100 µl samples of serum from all 10 healthy male subjects. The normal range was smaller in winter, the serum values in the 10 subjects varying from 0.2 to 0.8 nkatal/l (mean value of 0.4 nkatal/l). In autumn and summer the range increased, the respective serum values being 0.3–1.9 nkatal/l (mean 0.8 nkatal/l) and 0.4–2.0 nkatal/l (mean 0.9 nkatal/l) (Fig. 3).

The seasonal variation in serum tyrosinase values is analogous with that of urinary 5-S-cysteinyldopa excretion, which is greatest in the summer, intermediate in the spring and autumn, and least in the winter (14). It has also been shown experimentally that UV exposure increases urinary excretion and serum levels of 5-S-cysteinyldopa (15–18), suggesting cysteinyldopa production to be increased in UV-stimulated melanocytes. The seasonal variation in serum tyrosinase activity found in the present study suggests that stimulation of the epidermal melanocyte by UV exposure increases tyrosinase activity. Thus, the season of the year and local climatic conditions need to be taken into account in future studies.

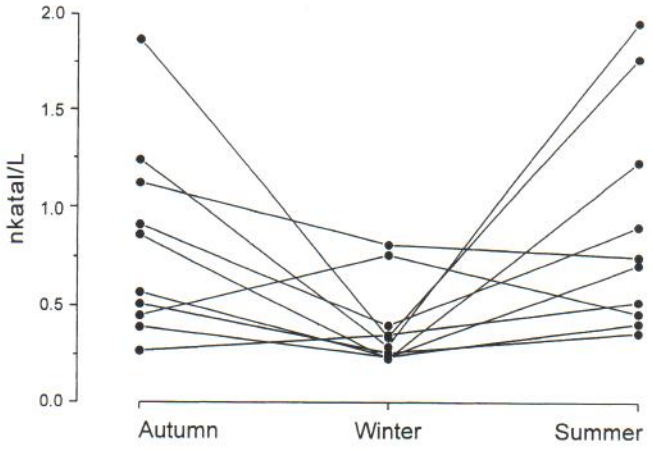

Fig. 3. Seasonal variation of serum tyrosinase activity in 10 healthy men.

Findings in previous studies have suggested that the tyrosinase activity found in the sera of melanoma patients may be due, at least partly, to enzymes released by living tumour cells (4, 8). The present findings suggest that the enzyme is also released from normal melanocytes.

L-tyrosine and L-dopa compete for the active site in human tyrosinase, and L-tyrosine functions as an inhibitor of dopa oxidase. Inhibition of dopa oxidation by 1 mM L-tyrosine (10) reduced 5-S-L-cysteinyl-L-dopa values to approximately one third of the values without tyrosine (Fig. 2). No significant reduction of the formation of 5-S-L-cysteinyl-D-dopa was seen after the addition of L-tyrosine.

The method described here made it possible to study the level of tyrosinase in human serum under physiological conditions. The seasonal variation we now report suggests that the serum level of tyrosinase activity is a sensitive indicator of melanocyte activity. The method will be useful in studies of the pigmentation system under normal, experimental and pathological conditions; its most important use will probably be in the follow-up of melanoma patients.

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