[CANCER RESEARCH 57, 5073–5076, November 15, 1997]

Prognostic Value of Serum 5-*S*-Cysteinyldopa for Monitoring Human Metastatic Melanoma during Immunochemotherapy[1]

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ABSTRACT

The melanin metabolite 5-*S*-cysteinyldopa (5-*S*-CD) has been reported to be helpful in detecting occult melanoma metastases and as a prognostic marker in B16 melanoma-bearing mice. The goal of our study was to analyze the significance of the serum 5-*S*-CD level for the biochemical detection of metastases in human malignant melanoma (MM) and for monitoring the progression or the immunochemotherapeutically induced regression of MM.

From 11 patients with metastatic MM observed between 1991 and 1995, serum samples were collected before and after each cycle of immunotherapy or immunochemotherapy. Samples were analyzed for 5-*S*-CD by automated high performance liquid chromatography with electrochemical detection.

Cycles of immunochemotherapy consisted of human interleukin 2 and IFN-α (four patients) or of human interleukin 2, IFN-α, and dacarbazine (seven patients).

Serum value of 5-*S*-CD in our normal controls was 1.9 ± 0.6 ng/ml. All patients with metastatic MM showed 5-*S*-CD serum levels above the upper normal limit of 3.2 ng/ml (10 nM) and ranged from 2.3-fold (4.3 ± 3.9 ng/ml) of the normal control values in early stages of metastases to more than 50-fold (94.3 ± 220.3 ng/ml) of the normal control values in advanced stages of the disease. In 28 of 41 (68%) immunochemotherapeutical cycles, a decrease of 5-*S*-CD was seen during therapy, and in 13 cycles (31.7%), an increase was seen. Patients with more than 68% decreasing cycles (defined as responders; $n = 5$) showed significantly longer survival times ($P = 0.008$) than patients with less than 68% decreasing cycles (nonresponders; $n = 6$). High levels of 5-*S*-CD were also observed in metastasizing amelanotic melanoma.

Serum 5-*S*-CD is a useful marker for monitoring the clinical course of MM patients, for discriminating between responders and nonresponders to immunochemotherapy, and as a prognostic factor concerning survival time and death risk.

INTRODUCTION

MM[3] incidence and mortality rates have significantly increased worldwide during the last decade, especially in Caucasians (1). In the United States, approximately 1 in 100 people born in the 1990s will develop MM during their lifetime (2). About 20% of these patients will develop metastases and die of widespread disease. The early detection of metastases and monitoring of disease progression can be crucial in these patients, for whom new immunotherapeutic and immunochemotherapeutic approaches may be implemented.

In addition to clinical and instrumental staging procedures like chest X-ray, sonography, CT, bone scintigraphy, magnetic resonance imaging, and whole-body positron emission tomography (3), several immunohistochemical and laboratory parameters have been reported to be of prognostic significance, *e.g.*, lactate dehydrogenase (4), neuron-specific enolase (5), 5,6DHI2C (6), 5-*S*-CD (6–14), immunohistochemical markers such as S-100 protein (15), NKI/C3 (15), HMB-45 (16), proliferating cell nuclear antigen (17), p53 (17), MIB 1 (18), and others.

Two types of melanin pigments, black eumelanin and reddish-brown pheomelanin, are produced in melanocytes and melanoma cells (19, 20). The key oxidative enzyme in the synthesis of the melanins, tyrosinase, catalyzes the oxidation of the amino acid tyrosine to dopa and then to dopa quinone (Fig. 1). Dopa quinone is either converted to eumelanin in a series of complex auto-oxidative reactions via dihydroxyindoles or alternatively reacts mainly with cysteine or glutathione and other steps of synthesis to form 5-*S*-CD, the major precursor of pheomelanin (20–23). Major portions of the melanin precursors 5-*S*-CD and dihydroxyindoles are oxidized to form the melanin pigments, but a minor portion also leaks into the blood stream (6) and therefore reflects melanin pigment synthesis.

Serum-5-*S*-CD, the most important and most specific secretion product of the melanocyte (24), is an excellent detector of metastasis and parameter of disease progression (8, 9, 24), showing significant elevation with the occurrence of metastasis and with the progression of the disease earlier than physical, radiological, or other laboratory investigations (6).

The purpose of our study was to analyze the serum 5-*S*-CD-level as a useful marker for monitoring tumor progression and regression due to immunochemotherapy in metastasizing MM. Furthermore, we wondered whether levels of 5-*S*-CD would provide information about the prognosis and the response to therapy.

PATIENTS AND METHODS

Patients. Eleven patients (seven males and four females; ages 28–77 years, mean, 47 years) with malignant MM from whom informed consent had been obtained were included in the study. The original diagnoses were amelanotic MM ($n = 2$), acrolentiginous MM ($n = 2$), superficial spreading melanoma ($n = 2$), nodular MM ($n = 2$), primary metastasizing MM ($n = 2$), and one MM without histology. All patients had documented lymph node (eight patients) or visceral (four patients) metastases or both (four patients; Table 1) when adjuvant immunochemotherapy was started. Four patients obtained cycles of adjuvant immunotherapy with IL-2 (EuroCetus GmbH, Frankfurt/Main, Germany) and IFN-α (Essex Chemie AG, Luzern, Switzerland), and seven patients' immunochemotherapy included IL-2, IFN-α, and DTIC (Miles Ltd. Dome Division, Slough, United Kingdom; Refs. 6, 25, and 26). Immunotherapy was given as eight cycles of 6 weeks, each applying IFN-α and IL-2. Immunochemotherapy consisted of a maximum of six cycles of DTIC, IFN-α, and IL-2, according to Dummer *et al.* (26).

Staging was performed every second or third cycle, following the 1987 Union International Contre Cancer tumor-node-metastasis classification. Staging procedures included chest X-ray, abdominal and lymph node sonography, and, in some cases, CT, bone scintigraphy, magnetic resonance imaging, and whole-body positron emission tomography (27).

Methods. Between 1991 and 1995, 41 serum samples from 11 patients with MM were collected before and after each cycle of immunochemotherapy. Treatment periods ranged from 1–13 months in individual patients. Control sera were obtained from 11 healthy subjects from August to September 1996. Blood samples without additives were centrifuged, and the collected sera were stored at −80°C until use.

5-*S*-CD was analyzed by automated high-performance liquid chromatogra-

Received 5/16/97; accepted 9/19/97.

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[1] Part of this work was presented as a poster at the 58th Meeting of the Society of Investigative Dermatology, April 23–27, in Washington, D.C., 1997.

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[3] The abbreviations used are: MM, malignant melanoma; 5-*S*-CD, 5-*S*-cysteinyldopa; IL, interleukin; CT, computer tomography; DTIC, dacarbazine.

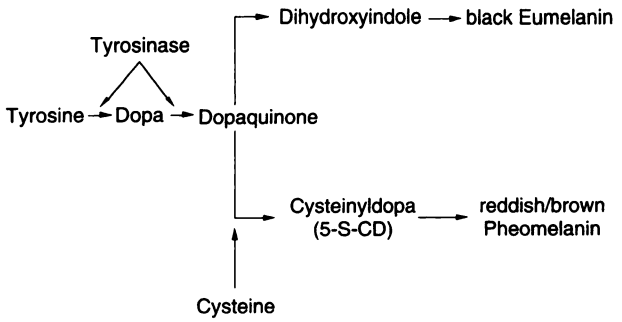

Fig. 1. Schematic presentation of melanogenesis.

phy with electrochemical detection. This method has been reported to be more sensitive and specific for 5-*S*-CD than the fluorometric method (7). In our hands, the reliable lower detection limit was between 1.0 and 2.0 ng/ml. Extraction and chromatography of serum samples were performed with a commercial kit (Immundiagnostik GmbH, Bensheim, Germany) according to the manufacturer's protocol. Sera were extracted and purified on acid-washed aluminum oxide, and then 50–100 μl of the extract were chromatographed on a reverse phase Nucleosil 120-5 column (Macherey-Nagel, Oensingen, Switzerland) with a flow rate of 0.7 ml/min using a Waters model LC 50 with an autosampler and a column heating set at 40°C (Waters Corp., Milford, MA). Detection occurred with a Waters model 464 electrochemical detector set at 750 mV with a sensitivity of 10 nA full scale. α-Methyldopa was used as an internal standard. Data reduction occurred with the program Baseline 810. Changes in 5-*S*-CD serum values of 0.2 ng/ml or more were taken as an increase or a decrease, respectively.

Statistics. The survival of responders and nonresponders was estimated by Kaplan-Meier curves and compared by the log-rank test. Relative risks were computed using the Cox regression model. Differences in serum levels between patients and controls were analyzed using the Mann-Whitney test. $P < 0.05$ was considered to be statistically significant. Data are presented as mean ± SD. Statistical analysis was performed using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Serum values of 5-*S*-CD in healthy controls (1.9 ± 0.6 ng/ml; range, 1–2.9 ng/ml) did not exceed the upper limit of the normal value of 3.2 ng/ml (10 nM; Ref. 6). Mean values of 5-*S*-CD before starting immunochemotherapy (4.3 ± 3.9 ng/ml; range, 1.3–13.3 ng/ml) and at the end of immunochemotherapy (94.3 ± 220.3 ng/ml; range, 0.9–730 ng/ml) were significantly (2.3 and 50.7-fold, respectively) higher than the mean values in our normal controls (1.9 ± 0.6 ng/ml; range, 1–2.9 ng/ml) as calculated by the Mann-Whitney test ($P = 0.04$ and 0.0007, respectively). Accordingly, median values were 1.7 (controls), 2.3, and 8.8 ng/ml, respectively (Fig. 2).

In the course of disease progression, there was a tendency for 5-*S*-CD levels to increase with increasing tumor burden due to lymphogenous and especially hematogenous metastases. The patient depicted in Fig. 3 showed a terminal increase after the end of the fifth therapy cycle (2.8 ng/ml 5-*S*-CD) up to the beginning of the sixth therapy cycle (8 ng/ml 5-*S*-CD), accompanied by the occurrence of multiple cervical, retrosternal, cardiophrenic lymphogenous metastases and peritoneal carcinosis with ascites. Analyzing the impact of lymphogenous or hematogenous metastases on 5-*S*-CD serum levels, striking differences in 5-*S*-CD increases in comparison to individual previous values were seen between lymphogenous (6.5 ± 11.8 ng/ml) *versus* hematogenous (52 ± 96.2 ng/ml) metastases.

From the 41 treatment cycles of immunochemotherapy analyzed before and after treatment, there was an average decrease of 5-*S*-CD serum levels to 65% in 28 cycles (68%) and an average increase to 161% in 13 cycles (32%). In six patients, decreasing and increasing values were both seen at different cycles during the treatment course.

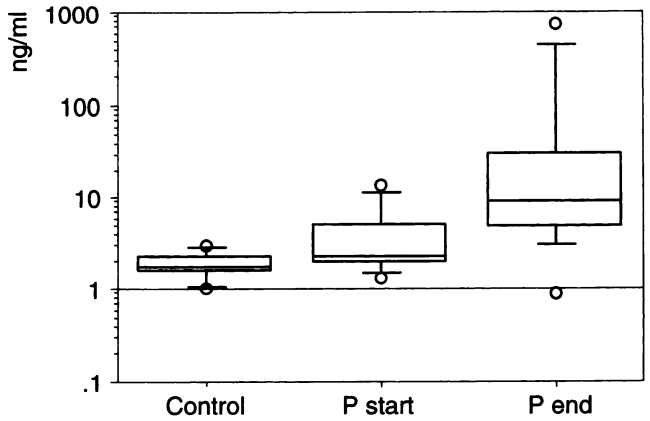

Fig. 2. Box plots of serum 5-*S*-CD levels from healthy controls (n = 11; mean, 1.86 ng/ml) and from patients at start of therapy (n = 11; mean, 4.26 ng/ml) and at the end of therapy (n = 11; mean, 94.26 ng/ml). The *boxes* contain the central 50% of data (25th–75th percentile) and the median = middle line. The extremely *short lines* indicate the 10th and 90th percentile, and the *points* indicate the extreme values. The mean patients' end value (94.26 ng/ml) is far above the highest 5-*S*-CD value of the control subjects (2.9 ng/ml). *P start*, start of therapy in patients; *P end*, end of therapy in patients.

Table 1 *Clinical data of patients with melanoma*

No.	Response to therapy	Sex	Age (yr)	Diagnosis	Site	Clark	Breslow (mm)	TNM[a]	Therapy	Survival (mo)
1	NR[b]	M	28	AmelM[c]	Ear	III	2.72	III	I[d]	25
2	NR	F	31	ALM[e]	Thigh	III	1.53	III	I	8
5	NR	M	35	ALM	Shoulder	V	6	III	IC[f]	21
7	NR	M	77	NM[g]	Upper arm	IV	2	II	IC	10
8	NR	M	42	AmelM	Nose	1)[h]	1)	1)	IC	5
9	NR	F	47	NM	Interscapular	IV	2.4	II	I	8
3	R[i]	M	48	SSM[j]	Shoulder	III	1.15	III	IC	99
4	R	F	40	SSM	Thigh	III–IV	2.2	III	IC	55
6	R	F	48	MM 1)	Vertex	1)	1)	1)	IC	33
10	R	M	72	ALM	Toe	III	2	II	IC	14
11	R	M	48	MM 2)[k]	Back	V	10.Apr	IV	I	41

[a] TNM, tumor-node-metastasis.
[b] NR, nonresponder.
[c] AmelM, amelanotic melanoma.
[d] I, immunotherapy (IL-2 and IFN-α).
[e] ALM, acrolentiginous melanoma.
[f] IC, immunochemotherapy (IL-2, IFN-α, and DTIC).
[g] NM, nodular melanoma.
[h] 1), excision without histology.
[i] R, responder.
[j] SSM, superficial spreading melanoma.
[k] 2), melanoma metastases of unknown primary tumor.

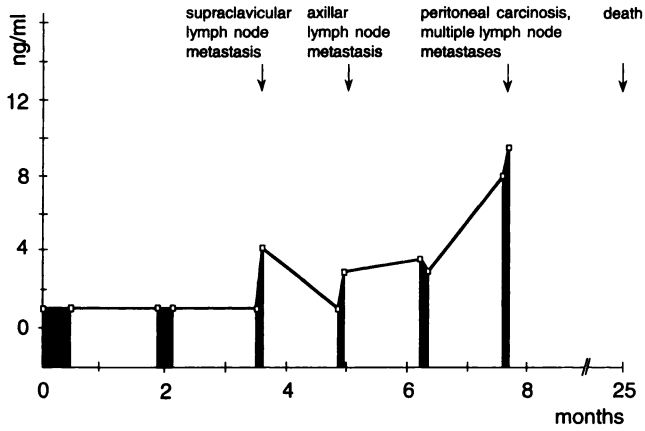

Fig. 3. The 5-S-CD course/development of a nonresponder during six cycles of immunochemotherapy is shown. There are no changes in 5-S-CD levels in the first two cycles, and there are three increases and one decrease during the therapy cycles. 5-S-CD ranged from <2.0 to 9.5 ng/ml. Survival time calculated from first diagnosis was 25 months. The patient had a primary melanoma at the right ear and already presented retromandibular and subpleural metastases before treatment. Before therapy, inguinal lymph node metastases and two hematogenous metastases in the right upper and middle lobe were observed. Each therapy cycle is indicated by a *hatched area*.

Patients showing treatment-dependent declines of 5-S-CD levels in more than 68% of their treatment cycles were referred to as responders (Fig. 3), and those with less than 68% were referred to as nonresponders (Fig. 4), respectively.

Responders showed significantly (P = 0.008) longer overall survival times as calculated from the time of diagnosis (48.4 ± 14.3 months; median, 41.0 months) than did nonresponders (mean values, 13.0 ± 3.25 months; median, 8.0 months; Fig. 5). Using the proportional hazard test, nonresponders had a 10.3 × higher death risk than responders at any time after diagnosis of melanoma.

When survival time was calculated starting from the initiation of immunochemotherapy or from the date of the occurrence of hematogenous metastases, responders again showed a 5.5× and 5.9× higher survival risk, respectively (P = 0.02 and 0.02, respectively), and a lower death risk than nonresponders.

DISCUSSION

Blood levels of 5-S-CD as determined in serum or plasma have been reported to be a reliable marker for monitoring the clinical course in MM (6, 11, 24, 28). Plasma concentrations of 5-S-CD correlate significantly with tumor weight of B16 melanoma in mice (29) and reflect tumor progression (30, 31). It has also been reported to be useful for the detection of metastases in early-stage melanoma in humans (11). Normally, levels in healthy persons do not exceed 3.2 ng/ml (10 nM; Ref. 6). Below this level, variations are described depending on the degree of tanning ability and sun exposure (24, 32) but not on race, skin type, or hair color (33). In comparison with urine samples, serum 5-S-CD analyses are claimed to be more precise due to their independence from age-related decrease of renal clearance, their narrower level variations, and an easier collection compared to 24-h urine samples (8). The mean serum value of 5-S-CD in our control group of 11 healthy Caucasians was 1.9 ± 0.6 ng/ml.

To our knowledge, there have been no investigations yet concerning the 5-S-CD levels in relation to therapy in a longitudinal study of patients with MM. MM patients without metastasis showed serum concentrations (mean value, 4.2 ± 1.6 nM, 1.3 ± 0.5 ng/ml) close to the upper limit of normal subjects (3.2 ng/ml, 10 nM; Ref. 6). When metastases occurred in our patients, 5-S-CD values significantly increased from 2.3- to more than 50-fold. In rare cases, however, serum levels may remain unchanged despite the development of metastases (10), as seen in 1 of our 11 patients (patient 1), in whom no change of 5-S-CD levels was seen during the first and second therapy cycles, even though subpleural and retromandibular lymph node metastasis occurred. If hematogenous metastasis occurred in the final periods of the disease, mean levels and variations of 5-S-CD levels in patients with end-stage hematogenous metastases were much higher (52.0 ± 96.2 ng/ml) than those in patients with lymphogenous metastases and lymph node involvement (6.5 ± 11.8 ng/ml; Fig. 2). The augmented levels of the melanoma precursor 5-S-CD in patients with hematogenous metastases are probably due to the presence of melanoma cells in the peripheral blood synthesizing and releasing this precursor. Another factor possibly influencing the 5-S-CD serum level is the localization of metastases as documented by patient 4, who, despite developing brain metastases, showed low levels of 5-S-CD even in the terminal stage, hence possibly indicating a high blood-brain barrier for 5-S-CD.

On the other hand, 5-S-CD levels seem to be independent of tissue pigmentation. Two cases of amelanotic melanoma (patients 1 and 8) also revealed an increase of 5-S-CD at the time of lymphogenous and hematogenous metastasis. The lack of pigmentation in amelanotic tissue might be explained by a later defect in

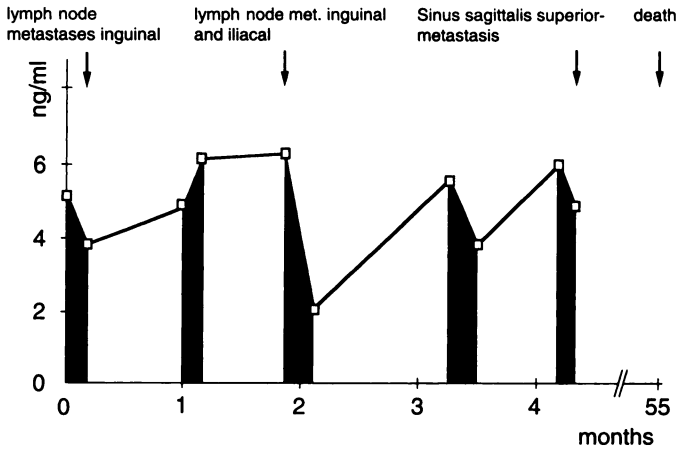

Fig. 4. The 5-S-CD course of a responder during five cycles of immunochemotherapy is shown. 5-S-CD increases once and decreases four times during the therapy cycles. 5-S-CD ranged from 2.0 to 6.3 ng/ml. Survival time calculated from the first diagnosis was 55 months. *Hatched areas*, therapy cycles.

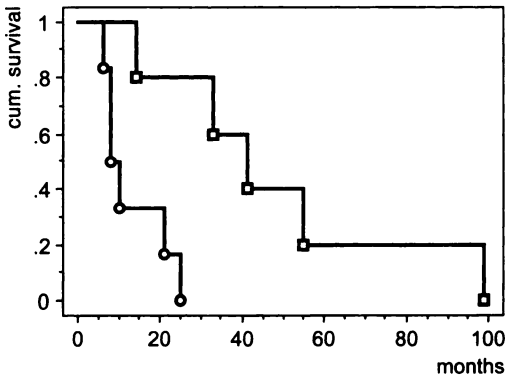

Fig. 5. Kaplan-Meier estimates of survival of responders and nonresponders from the beginning of diagnosis are shown. X axis, the survival time in months; Y axis, the cumulative survival time with probability between 0 and 1. □, responders; ○, nonresponders.

the melanogenesis pathway after the formation of 5-*S*-CD, without affecting 5-*S*-CD synthesis itself. This is in accordance with findings of Peterson *et al.* (11), showing that plasma 5-*S*-CD levels in patients with amelanotic melanoma are not significantly different from those with pigmented melanomas. Furthermore, albino mice are reported to excrete as much 5-*S*-CD as black mice (13, 24), and plasma 5-*S*-CD concentrations in tyrosinase-positive and -negative albino patients were found to be almost equal to control values (34).

Studies in B16 mice have demonstrated that plasma 5-*S*-CD may be a useful marker for evaluation of the efficacy of immunochemotherapy (25). Similar studies in humans have not yet been reported. In our patients, serum 5-*S*-CD values depended on both the increase of the tumor burden and the immunochemotherapy, as assessed by measuring serum levels immediately before and after each immunochemotherapeutic cycle. A significant decrease of 5-*S*-CD was seen in 68% of the 41 immunochemotherapeutic cycles. In patient 11 (Table 1), a shift from an elevated precycle (7.3 ng/ml) to a normal postcycle (2 ng/ml) 5-*S*-CD was paralleled by a dramatic regression of liver metastasis as demonstrated by a CT scan. Nevertheless, in 32% of all cycles, an increase of serum 5-*S*-CD was found. The increase of 5-*S*-CD during therapy may be due to the release of intracellular 5-*S*-CD from tumor cells undergoing necrosis due to immunomodulating cell destruction under immunochemotherapy. Other modalities of cell damage, like ultraviolet B, also induce excretion of 5-*S*-CD that is due to melanocyte damage rather than to increased melanin synthesis (32, 33). No significant difference between immunotherapy with IL-2 and IFN-α or immunochemotherapy with IL-2, IFN-α, and DTIC was observed. Patients were subdivided into two groups. Responders showed a drop of 5-*S*-CD serum values in at least two-thirds (68%) of their immunochemotherapeutic cycles, whereas nonresponders consisted of patients exhibiting a decrease in less than 68% of their cycles. Survival times were significantly higher in responders than in nonresponders (Fig. 5), irrespective of whether these were calculated from time of diagnosis ($P = 0.008$), from the start of therapy ($P = 0.02$), or from the time of the first occurrence of hematogenous metastasis ($P = 0.02$); therefore, death risks were significantly higher in nonresponders (9.4, 5.5, and 5.9 times higher, respectively).

In conclusion, longitudinal studies of 5-*S*-CD serum levels in patients with malignant MM are an easy-to-perform reliable measurement for monitoring the clinical course of the disease, for the early detection of metastases, and for monitoring immunochemotherapy, with special emphasis on the tumorolytic effect of the therapy. Furthermore, this monitoring allows discrimination between responders and nonresponders and hence yields prognostic information.

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Cancer Res 1997;57:5073-5076.