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Advances in Brief

Melanoma-inhibiting Activity, a Novel Serum Marker for Progression of Malignant Melanoma[1]

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Abstract

Melanoma-inhibiting activity (MIA) was isolated previously as a small soluble protein secreted from malignant melanoma cell lines *in vitro*. *In vivo*, highly restricted expression patterns in melanocytic tumors were identified. We therefore quantitated serum levels of MIA protein by means of a nonradioactive ELISA and investigated whether MIA provides a clinically useful parameter in patients with malignant melanomas. Here, we report enhanced MIA serum levels in 13 and 23% of patients with stage I and II disease, respectively, and in 100% with stage III or IV disease. Compared with S-100 and soluble intercellular adhesion molecule 1 serum levels in these patients, MIA was the most sensitive marker. Response to therapy in stage IV disease correlated with changes in MIA serum levels. Measuring repeatedly sera of 350 patients with a history of stage I or II melanoma during follow-up, we detected 32 patients developing positive MIA values. At the time of serum analysis, 15 of them had developed metastases, and one presented with metastatic disease 6 months later. In contrast, none of the patients with normal MIA serum levels developed metastases during the follow-up period of 6–12 months. In conclusion, MIA represents a novel serum marker for systemic malignant melanoma revealing the highest sensitivity and specificity among currently available markers. Useful clinical applications include staging of primary melanomas, detection of progression from localized to metastatic disease during follow-up, and monitoring therapy of advanced melanomas.

Introduction

MIA[3] has been identified previously within growth-inhibitory activities purified from tissue culture supernatant of malignant melanoma cells *in vitro* (1, 2). A single copy gene coding for a secreted M_r 11,000 protein was cloned and mapped to human chromosome 19q13.32–13.33 (3, 4). MIA mRNA was identified independently by a differential display approach comparing differentiated and dedifferentiated cartilage cells *in vitro* and has been referred to as CD-RAP (5).

Analyses of embryonic and adult tissues have revealed a highly cell type-restricted expression pattern of MIA mRNA. In nonneoplastic tissues, MIA was detected mainly in developing and mature cartilage (5, 6). In malignant tumors, high MIA mRNA levels were detected in almost 100% of malignant melanoma samples (7, 8). Comparing the expression in normal skin, benign human skin melanocytes, benign melanocytic nevi, and malignant melanomas, we observed that MIA mRNA levels parallel progressive malignancy of melanocytic tumors (7). Encouraged by these results and the fact that MIA is a small, soluble, and secreted protein, we established an ELISA to quantitate MIA protein levels in serum. The ELISA was used to investigate whether MIA serum levels provide prognostic information in patients with malignant melanomas.

Materials and Methods

Patients. Serum samples were analyzed from 112 patients with malignant melanoma (38 with stage I, 13 with stage II, 6 with stage III, and 44 with stage IV) obtained prior to surgery and from 350 clinically tumor-free patients obtained during a follow-up period of 6 to 12 months after removal of a primary stage I or II melanoma 6 months to 5 years previously. Diagnosis was based on histological examination in all cases and confirmed by two independent histopathologists and S100 and HMB45 immunohistochemistry in all doubtful cases. Tumor stage, patients' sex and age, and histological growth pattern are summarized in Table 1. Tumor thickness was determined according to Breslow (9) and tumor stage according to the American Joint Committee on Cancer (stage I, T_{1-2}, N_0, M_0; stage II, T_{3-4}, N_0, M_0; stage III, T_{1-4}, N_{1-2}, M_0; and stage IV, T_{1-4}, N_{1-2}, M_1; Ref. 10).

Furthermore, serial serum samples from five patients during chemotherapy of stage IV melanoma were analyzed. Response to therapy was followed by clinical examination, routine laboratory tests, and chest X-rays, and tumor size measurements were taken by ultrasound and computerized axial tomography scans as well as nuclear magnetic resonance imaging. Sera of five additional patients were measured before and after surgery of metastatic melanomas.

A reference panel of 72 sera from healthy blood donors (age range, 19–86 years; mean ± SD, 43.9 ± 19.2 years) was selected based on the following criteria: no use of medication, no record of any metabolic disorder, and no record of a malignant tumor. Additional controls included sera from 50 patients with sepsis, from 23 patients with brain tumors (WHO grades III and IV gliomas), and from 243 patients with advanced epithelial and mesenchymal tumors (all in stage III or IV; summarized in Table 2).

All sera were stored at −80°C until assayed and determined blind of clinical information.

Detection of MIA, S100, and sICAM-1 in Serum. MIA was measured by a one-step ELISA. Two MABs directed against 14-meric NH_2-terminal and COOH-terminal peptides (MAB 1A12 and MAB 2F7; Boehringer Mannheim, Mannheim, Germany) were raised and conjugated to horseradish peroxidase and biotin, respectively. Ten μl of serum or standard were incubated with 200 μl reagent containing MAB-biotin (2F7) and MAB-horseradish peroxidase (1A12) in streptavidin-coated 96-well plates for 45 min with shaking. After washing three times with PBS, 200 μl of 2,2′-azino-di-(3-ethylbenz-thiazoline sulfonate) (Boehringer Mannheim) were incubated in the wells for 30 min and measured colorimetrically at 405 nm. Using standard concentrations of recombinant MIA purified from stably transfected Chinese hamster ovary cells, we measured linear signals at MIA concentrations between 0.1 and 50 ng/ml. Reproducibility of test results was confirmed by measuring repeatedly eight standard sera using different ELISA lots (mean SD, 9.4%). All serum samples and standards were measured in duplicate, and results never varied more than 5%. The standard curve was calculated in a linear fashion.

S100 was measured by a commercially available immunoradiometric assay (Byk-Sangtec Diagnostics, Dietzenbach, Germany; Ref. 11) following precisely the manufacturer's instructions and using the provided internal standard

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[3] The abbreviations used are: MIA, melanoma-inhibiting activity; sICAM, soluble intercellular adhesion molecule; MAB, monoclonal antibody.

Table 1 *Stage of disease, sex, age and histological growth pattern of the primary tumour in 91 patients with malignant melanoma*

	Stage[a]	I	II	III	IV
n (101)		38	13	6	44
Male:Female		19:19	6:7	4:2	20:24
Age		56.8 ± 14.8	56.1 ± 20.8	50.8 ± 14.4	54.1 ± 13.5
Growth pattern	SSM	28	11	5	33
	NMM	3	1	1	9
	ALM	2	1		2
	LMM	5			

[a] SSM, superficial spreading melanoma; NM, nodular melanoma; ALM, acral lentiginous melanoma; LMM, lentigo maligna melanoma.

reagents. sICAM-1 was measured using a commercially available one-step ELISA kit (Boehringer Mannheim).

Results

Enhanced MIA Serum Levels in Patients with Metastatic Malignant Melanoma. MIA serum levels in a reference group of 72 healthy blood donors varied between 1.8 and 7.6 ng/ml following a Gaussian distribution (data not shown). Values above the 95th percentile of 6.5 ng/ml were defined positive. Analyzing groups of healthy donors at ages 0–30, 31–60, and above 60 years separately revealed that MIA serum levels in healthy people do not depend on age (Table 2). Analyzing the additional sera from 50 septic patients hospitalized at our intensive care unit, we examined whether MIA serum levels rise in response to activation of the immune system. Serum levels of septic patients ranging from 1.4–6.8 ng/ml were nearly identical to values obtained from healthy donors. A summary of all measurements is shown in Table 2.

In contrast, all sera obtained from 50 patients with metastatic melanomas (stage III and IV) were positive (between 7.1 and 82.0 ng/ml), whereas only 13% of sera from 38 patients with stage I melanomas and 23% of sera from 13 stage II patients were positive. MIA levels were normal in all nine patients who had surgical removal of common benign nevi and raised slightly in one patient with numerous melanocytic nevi ("multiple nevi syndrome") who had surgery of a benign melanocytic nevus (8.1 ng/ml). Sixteen % of sera from 25 patients with basal cell carcinoma, a lesion usually arising in sun-damaged skin, had slightly increased MIA levels (2.7–8.4 ng/ml). Measurements from patients with melanomas at stages I–IV in comparison to healthy donors are detailed in Fig. 1*A*. In summary, we conclude that MIA serum levels provide a sensitive surrogate marker for detection of metastasized melanomas. Even at a cutoff of 7.7 ng/ml, resulting in a 0% false-positive rate in our healthy control group, 83 and 95% sera of patients with stage III and stage IV disease score positive.

Addressing the specificity of MIA as a melanocytic tumor marker, we measured MIA in a large series of 270 samples obtained from patients with advanced stage III–IV epithelial or mesenchymal tumors and high-grade gliomas (WHO grade III or IV). Positive values were measured in 16% of patients with carcinoma of the ovary, 17% of patients with pancreatic carcinomas, 14% of patients with breast cancers, and 7% of patients with colon carcinomas, but all sera from patients with gliomas, small cell cancer of the lung, and sarcomas were negative (Table 2). Performing reverse transcription-PCR analyses, we were able to demonstrate MIA mRNA expression in some breast and colon cancer specimens, and therefore, we can rule out that positive ELISA measurements in these tumors result from nonspecific binding to mucin secreted from these tumors (data not shown). Thus, MIA values were raised in a small percentage of patients with advanced epithelial neoplasms.

Sensitivity and Specificity of MIA, S100, and sICAM-1 Serum Levels. S100 and sICAM-1 serum levels have been correlated previously with melanoma progression (11–13). Comparing sensitivity and specificity of MIA serum levels with these markers, we measured S100 levels in all available melanoma and reference sera and sICAM-1 levels in a subset of sera. The cutoff value for S100 was set to 0.15 ng/ml according to previously published results (12). Only 30 of our 49 sera from stage III and IV patients were positive for S100, and all stage I and II sera were negative (Fig. 1*B*). In the control groups, 4% of sera from healthy donors, 20% from septic patients, 16% from patients with gliomas, and 5% from patients with advanced carcinomas scored positive for S100.

In contrast to MIA and S100, discrimination between melanoma patients, septic patients, and healthy donors based on sICAM-1 levels was very poor. Setting a cutoff value at 280 ng/ml, 33% of healthy donors, 43% of patients with stage III or IV melanomas, 17% of patients with basal cell cancers, 66% of patients with advanced gliomas, and 71% of septic patients revealed positive serum sICAM-1 levels. Similar disappointing results with respect to discrimination between healthy donors and patients with metastatic malignant melanoma or inflammatory diseases have been reported previously (14). Thus, we did not complete sICAM-1 measurements in all our patients.

These data indicate that MIA as a serum marker for systemically

Table 2 *Number of samples, median, and range of MIA serum levels (ng/ml), and percentage of positive samples*

Patients	*n*	Median	Range	No. positive	% positive
Healthy	72	3.6	5.8 (1.8–7.6)	2 of 72	
Healthy, <30 years	37	3.6	5.8 (1.8–7.6)	1 of 37	
Healthy, 30–60 years	21	4.0	3.7 (1.9–5.6)	0 of 21	
Healthy, >60 years	14	3.8	4.3 (3.1–7.4)	1 of 14	
Sepsis	50	2.5	5.4 (1.4–6.8)	1 of 50	2
Metastatic melanoma (stage III or IV)	50	18.8	77.4 (7.1–8.2)	50 of 50	100
Basal cell carcinoma	25	4.2	5.7 (2.7–8.4)	4 of 25	16
Melanocytic nevus	10	3.8	6.0 (2.1–8.1)	1 of 10	10
Brain tumor	23	2.6	2.6 (2.0–4.6)	0 of 23	0
Small cell cancer of lung	3	2.6	2.1 (2.5–4.6)	0 of 3	0
Ovarian cancer	31	5.6	29.7 (3.9–33.6)	5 of 31	16
Pancreatic cancer	34	5.3	14.7 (3.7–18.4)	6 of 34	17
Breast cancer	78	4.97	9.3 (2.6–11.9)	11 of 78	14
Colon cancer	82	4.9	6.5 (2.6–8.9)	6 of 82	7.3
Sarcomas	19	4.6	4.2 (1.4–5.6)	0 of 19	0

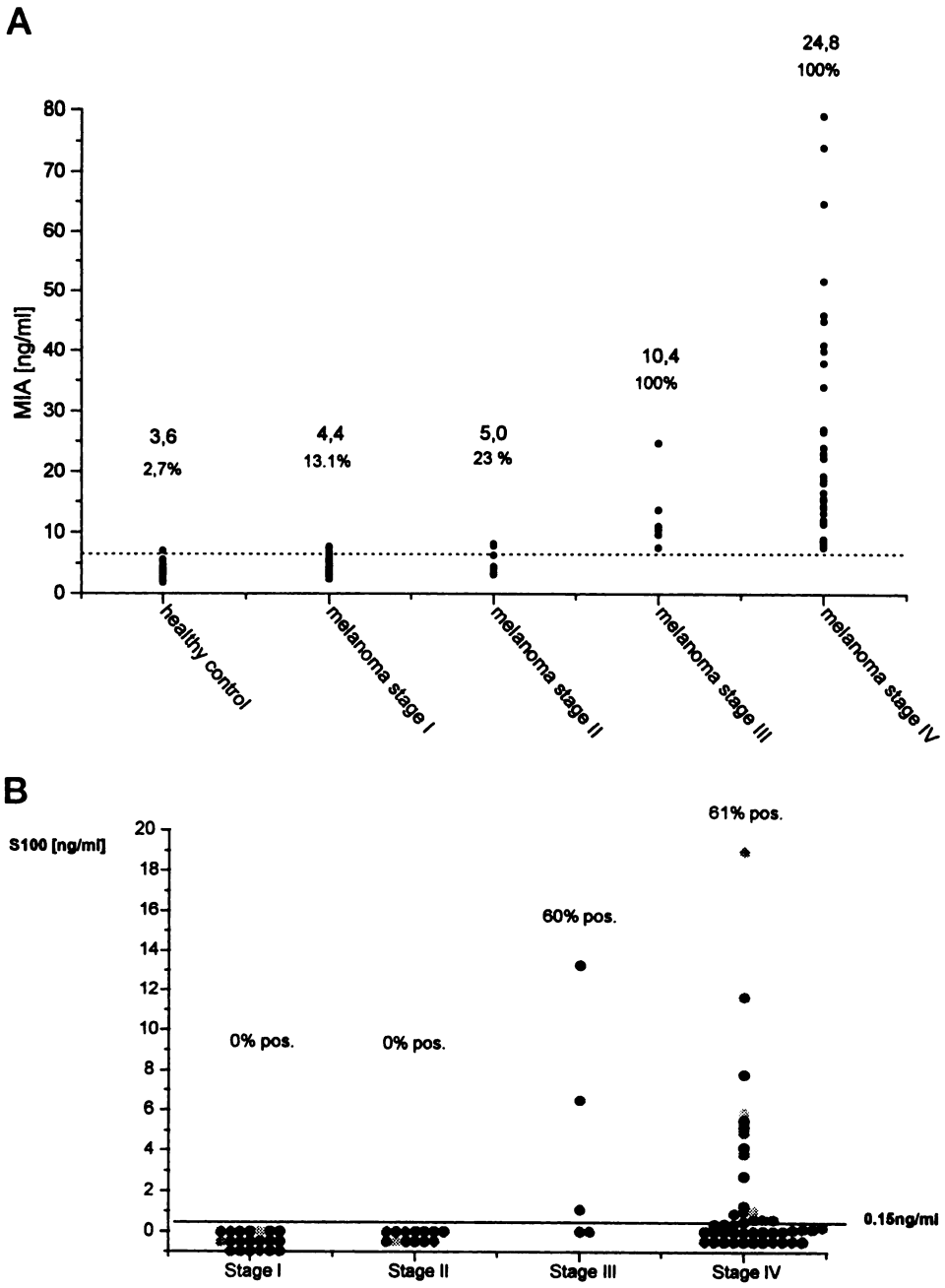

Fig. 1. *A*, MIA serum levels in healthy blood donors and patients with malignant melanoma of different stages. Median values and percentages of positive sera are indicated (*top*). *Dashed line*, cutoff level (6.5 ng/ml). *B*, S100 serum levels in 82 of the 101 patients with malignant melanoma that were tested for MIA. *Line*, cutoff level (0.15 ng/ml). Stage I, $n = 20$; stage II, $n = 13$; stage III, $n = 5$; stage IV, $n = 44$.

progressed malignant melanoma has a specificity comparable with S100. However, enhanced MIA serum levels represent a significantly more sensitive parameter for malignant melanoma at all stages when compared to S100 values. Both serum markers, MIA and S100, are clearly more specific than sICAM-1 levels.

MIA Serum Levels in Patients with Primary Malignant Melanomas. As detailed in Fig. 1*A*, 13% of patients with stage I and 23% of patients with stage II melanomas presented with MIA serum levels above the 95th percentile of healthy blood donors. Therefore, a subgroup of primary melanomas can be defined based on positive MIA serum levels. Analyzing histological information of these tumors, we did not detect a clear correlation between tumor thickness and MIA values (data not shown). Regression analysis revealed a correlation coefficient $r = 0.2357$, indicating that the Breslow index and MIA serum level may represent independent tumor parameters.

Monitoring MIA Serum Levels during Therapy of Malignant Melanoma. Furthermore, a possible correlation between total tumor burden and MIA serum levels in some of our melanoma patients was analyzed. In eight patients with stage III or IV melanomas undergoing surgical removal of metastases, we had the opportunity to compare MIA levels obtained immediately prior to surgery and within 2 weeks after surgery. MIA levels dropped significantly in all five cases (between 21 and 72%; mean reduction of 47%). In three cases, MIA levels dropped to normal, and these patients were clinically free of metastasis 3 months after surgery (data not shown).

Furthermore, serial MIA measurements were obtained from six patients undergoing combined surgical and chemotherapy of stage IV melanoma. Three patients with rapidly progressive disease unresponsive to treatment (Fig. 2, *P1–P3*) died before completing therapy and showed rising MIA levels. However, a reduction in MIA levels was measured in two patients responding to therapy (Fig. 2, *P4* and *P5*).

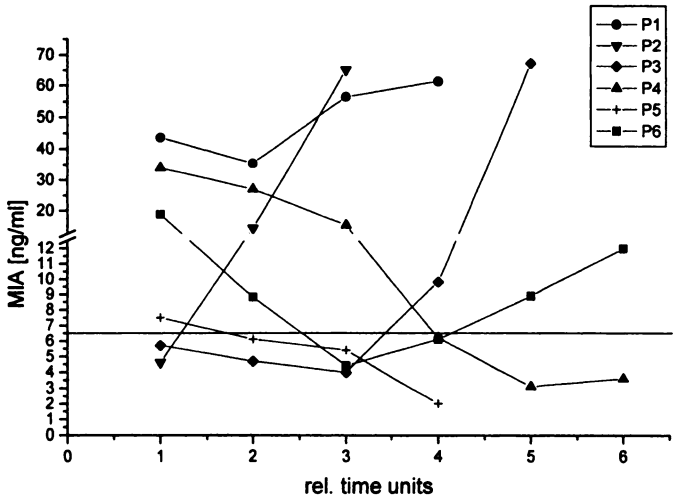

Fig. 2. MIA serum levels in six patients (*P1–P5*) during therapy for stage IV disease. Time scale from P1 to P6: P1, 6 months; P2, 15 months; P3, P4, and P6, 12 months; and P5, 9 months.

Response was assessed by elimination of all tumor nodules as determined by X-ray, ultrasound, and computerized axial tomography and nuclear magnetic resonance scans. In addition, patient P6 successfully completed therapy, but very soon the disease relapsed and progressed significantly, paralleling closely a rise in MIA levels. Taken together, these results indicate that changes in total tumor burden during therapy or tumor progression can be monitored by measuring MIA serum levels.

Detection of Metastasis during Follow-up of Melanoma Patients. Next, we analyzed the potential clinical significance of MIA serum levels in detecting disease progression of patients with surgically excised stage I or II melanomas. Sera of 350 clinically healthy patients obtained at 6-month intervals at the outpatient departments of our clinics in Regensburg and Munich were measured. All patients had a history of surgical removal of a malignant melanoma between 6 months and 5 years earlier but had no record of any metastatic disease thereafter. At every visit, patients were examined clinically for occurrence of metastatic disease, and a serum specimen was taken in parallel. Thirty-two patients presented with enhanced MIA serum levels; 15 of these patients had developed metastases based on clinical examination performed in parallel with MIA measurements. One additional patient presented with lymph node metastasis 6 months later, indicating that a rise in MIA serum levels may precede clinically detectable metastatic disease. Lymph nodes or tumor nodules were excised in all 16 patients, and the diagnosis was confirmed by histological examination. The other 16 patients presented free of clinically detectable disease. These patients will be monitored additionally to determine the significance of enhanced MIA values as an early indicator of disease progression. Importantly, no signs of tumor progression were detected in all 318 patients presenting with normal MIA values.

Discussion

Results presented in this study define MIA as a novel serum marker with clinical significance in staging and monitoring metastatic melanomas. Previous analyses performed by others and ourselves (7, 8) have shown that MIA is strongly expressed and secreted from malignant melanoma cells and at low levels from some benign melanocytic nevi but not from normal skin melanocytes. Preliminary reverse transcription-PCR amplifications of RNA extracted from a panel of different malignant tumor samples have revealed a high specificity of MIA expression in melanocyctic tumors (15). Establishing a nonradioactive MIA-ELISA, we now show that MIA serum levels are raised above the 95th percentile of healthy control donors in all 50 patients with clinically apparent metastatic melanoma. Furthermore, of 350 patients in the follow-up program, all 16 of those who progressed from localized to metastasized melanomas presented with enhanced MIA values. In contrast, none of the patients with negative MIA values developed metastasis. Thus, a rise of MIA serum protein levels during tumor follow-up is a significant surrogate marker for disease progression. Because quantitation of MIA by a nonradioactive ELISA represents a simple and economic laboratory test, it may contribute to saving more expensive and invasive diagnostic procedures. Additional extension of our follow-up studies is warranted to determine whether MIA provides a marker of disease progression in all cases and whether the 16 patients who developed positive MIA values but had no clinical signs of disease progression will develop metastases at a later stage.

Comparing MIA, S100, and sICAM-1 levels in patients with stage III or IV melanoma, we found a high specificity of both S100 and MIA for detection of advanced melanomas. However, the sensitivity of MIA in detecting melanomas of all stages was significantly higher than S100. Consistent with previous reports, sICAM-1 levels seem of little clinical significance in detection of melanomas (14).

Interestingly, we did not observe a strong correlation between Breslow indices and MIA levels in patients with stage I and II melanomas, suggesting that the rate of MIA secretion from malignant melanomas does not solely depend on the total tumor mass. The tumor thickness has been well established as the single most predictive parameter for disease progression of primary melanomas (16). Our results raise the possibility that MIA represents a prognostic marker independent of tumor thickness, providing additional diagnostic information. Clearly, a larger prospective study is needed to compare the significance of MIA values with established tumor parameters.

In summary, we conclude that MIA is of clinical value in both staging of primary melanomas and detecting tumor progression during follow-up and also in monitoring therapy of advanced disease. Additional evaluation is needed addressing possible clinical significance as an independent prognostic marker of stage I and II disease.

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