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The urokinase plasminogen activator and its inhibitors PAI-1 and PAI-2 in primary cutaneous melanoma

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Background. *We investigated the differences in urokinase plasminogen activator (uPA) and its inhibitors type1 and 2 (PAI-1/2) concentrations in clinically suspected nevi, primary cutaneous melanoma and normal skin and correlations with histopathological prognostic factors of primary melanoma.*
Patients and methods*. Fifty-one patients were enrolled. The tissue concentrations of uPA, PAI-1 and PAI-2 were quantified by enzyme-linked immunosorbent assay (ELISA).*
Results. *Mean uPA and PAI-1 concentrations in melanomas were higher than in normal surrounding skin (uPA: 1.08; vs. 0.48 ng/mgp; PAI-1: 14.07 vs. 2.07 ng/mgp; $p < 0.001$). uPA and PAI-1 concentrations were higher in melanomas than in nevi, and higher in nevi than in normal surrounding skin (uPA: $p > 0.05$; PAI-1: $p = 0.02$). PAI-2 concentration was higher in normal surrounding skin than in nevi and melanomas ($p > 0.05$). Melanoma uPA, PAI-1 and PAI-2 concentrations correlated significantly with normal skin ($r = 0.73, 0.54, 0.38$ respectively). PAI-1 was significantly lower in melanomas of Breslow thickness ≤ 0.75 mm, Clark invasion of 0+I, without microscopic ulceration, without vascular invasion ($p < 0.01$) than in melanomas of Breslow thickness > 0.75 mm, Clark invasion > II, with ulceration and vascular invasion.*
Conclusions. *Determination of uPA and PAI-1 can provide significant additional prognostic information for melanoma patients.*

Key words: skin neoplasms - melanoma; urokinase plasminogen activator; plasminogen activator inhibitor 1; plasminogen activator inhibitor 2; prognosis

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Introduction

Malignant melanoma is one of the most aggressive human tumours. The prognosis of melanoma as well as other cancers is mainly dependent on its ability to invade and metastasise. Prognostic markers such as Breslow tumor thickness, Clark level of invasion, ulceration and vascular invasion are used for defining melanoma patients at risk and fol-

lowing adjuvant therapy. The clinical importance of other factors (clinical, biochemical-molecular) has not been established yet.[1]

Cancer invasion and metastasis are multistep events involving local invasion of the extra cellular matrix, angiogenesis, invasion of the blood vessel wall, survival of malignant cells in the vascular system, extravasation, and establishment of a secondary growth. During most of these steps, extra cellular matrix and basement membrane have to be degraded.[2] The breakdown of these barriers is catalysed by the proteolytic enzymes, which are released from the invading tumour. Four different proteases are mainly involved: metalloproteinases (collagenases, stromelysin, gelatinases), cysteine proteinases (cathepsin B, H, L), aspartyl proteases (cathepsin D), serine proteases (plasminogen activation system).[3]

The two known plasminogen activators (PAs) are tissue type (tPA) and urokinase type plasminogen activator (uPA). Proteolytic activity of tPA is important for the degradation of intravascular blood clots, while uPA contributes to extracellular proteolysis in a wide variety of physiological and pathological processes.[2-4] uPA has many activities. It converts inactive plasminogen to plasmin, which degrades most substrates in the extracellular matrix (proteoglycans, laminin, fibronectin, vitronectin), and activates other proteases (procollagenases, uPA). uPA is activated also by kallikrein, trypsin, cathepsin B, L, thermolysin and nerve growth factor.[3,4] Beside these degradative functions uPA exerts other activities that may enable it to play a role in invasion and metastasis. These include stimulation of cellular proliferation, enhancement of cellular migration, alteration of cellular adhesive properties and activation of specific growth factors.[3-5] Active uPA binds to a membrane-bound receptor known as u-PAR. uPA activity is controlled by two inhibitors, plasminogen activator inhibitor type - 1 (PAI-1) and type - 2 (PAI-2). In addition to inhibiting uPA, PAI-1 modulates cellular adhesion and migration by its attachment to extracellular protein vitronectin. PAI-2 is important in inhibition of apoptosis.[3,4] A strong impact of these proteolytic factors on prognosis of the cancer disease has been observed in a variety of malignancies. The strongest and most consistent evidence of a prognostic role exists with breast cancer. Elevated concentrations of uPA, PAI-1 and uPAR are associated with poor prognosis, high levels of PAI-2, on the other hand, correlate with good outcome.[2,5] uPA is prognostic also in gastric, colorectal, oesophageal, renal, endometrial, and ovarian cancer.[2,4,5] It is well known, that uPA, PAI-1 and PAI-2 expression in human melanoma cell lines correlated with a high metastatic capacity in nude mice.[6] However in human cutaneous and uveal melanoma uPA, PAI-1 and PAI-2 had not been detected in early stage of melanoma but appeared frequently in advanced primary melanoma and melanoma metastatic lesions.[7,8]

Our study was aimed to find out the differences of uPA, PAI-1 and PAI-2 concentrations in clinically suspected nevi, primary melanoma and normal skin concentrations and their correlation to the most important histopathological prognostic factors: Breslow thickness, Clark invasion, ulceration and vascular invasion.

Patients and methods

Patients

Fifty-one patients with clinical confirmed primary cutaneous melanoma (27 women: mean age 49.7; range 21-84 years and 24 men: mean age 56.5; range 17-83 years) were enrolled into a prospective study between 1998-2000. Inclusion criteria were: absence of metastases and macroscopically and histological complete surgical removal of the primary cutaneous melanoma (UICC pT1or T2N0M0, AJCC stage I and II).[9] The Medical Ethics

Committee at the Ministry of Health of the Republic of Slovenia approved the study protocol.

We totally excised melanoma lesions with safety edge of 1-2 cm. We excised 2 x 2 x 2 mm tissue specimens of the lesions and of the normal skin (at least 2 cm far away from the edge of tumours) for the quantification of uPA and PAI-1/2. They were snap-frozen in liquid nitrogen and stored at - 80°C.

The remaining tissue was fixed in 10% formalin and embedded in paraffin for histological examination. The histological diagnosis in 8 patients was dysplastic nevus and in 43 primary cutaneous malignant melanoma with clinical stage I (T1-2 N0 M0) less than 1.5 mm thick. The clinical and histopathological characteristics of primary tumours are shown in Table 1.

Tissue extraction and ELISA for uPA, PAI-1 and PAI-2

The uPA concentrations were determined in 41 pairs of triton extracts, and the PAI-1 and PAI-2 concentrations in 51 pairs of cytosols prepared from tumour and adjacent normal tissue samples (matched pairs) weighing 50 mg, obtained at surgery. The still frozen cut sections were dipped into liquid nitrogen and then pulverized in a microdismembrator (Braun - Melsungen, Melsungen, Germany).

For the triton extracts the still frozen pulver was dispensed with Tris buffered saline (TBS) (0.02 M Tris-HCl, 0.125 M NaCl, pH 8.5) containing 1% non-ionic detergent Triton X-100 (Sigma, St. Louis, Missouri, U.S.A.). The suspension was gently shaken for 3 hours at 4°C. For the cytosol the still frozen pulver was dispensed in a phosphate buffer (5 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 1 mM monothioglycerol, 10% (vv-) glycerol, pH 7.4). Both, the tissue extracts and cytosol suspension were subjected to ultracentrifugation (100 000 g/45 min at 4°C) to separate tissue debris. Supernatants were collected, divided into aliquots and stored at - 70°C until use.

uPA, PAI-1 and PAI-2 concentrations were determined by commercially available ELISA kits (American Diagnostica, Inc., Greenwich, U.S.A.) for uPA, PAI-1 and PAI-2. Details of the kits are described elsewhere.[10] Levels of the uPA, PAI-1 and PAI-2 are expressed in ng/mg proteins. Protein content was determined with Bio Rad method.

Statistical analysis

Statistical analysis was performed using the SPSS for Windows program. Differences of uPA, PAI-1 and PAI-2 concentrations in melanomas, nevi and normal skin were analysed by the Wilcoxon test. Spearman's

Table 1. Clinical and histopathological characteristics of the tumours

Characteristics	Number
Dysplastic nevus	8
Melanoma	43
Localization of primary lesion	
Head	4
Trunk	34
Extremities	13
Histopathological characteristics of melanomas	N = 43
Breslow	
≤ 0,75	28
> 0,75	15
Clark	
0+I	12
II + III	31
Ulceration	
Yes	7
No	36
Vascular invasion	
Yes	3
No	20
Undetermined	20
Histopathological type	
Lentigo maligna	2
Superficial spreading	35
Nodular	2
Unclassified	4

rank correlations were evaluated for the relations between uPA, PAI-1 and PAI-2 concentrations in melanomas and normal skin. Differences of uPA, PAI-1 and PAI-2 concentrations in melanomas and histomorphological variables among various groups of patients were analysed by the two-tailed t-test and the analysis of variance (ANOVA).

The p-values ≤ 0.05 were considered significant.

Results

uPA, PAI-1 and PAI-2 concentrations in melanomas, nevi and normal skin

Among 51 tumour specimens (43 melanomas, 8 dysplastic nevi) and normal skin, the concentration of u-PA was determined in 41 pairs of triton extracts (36 melanomas, 5 nevi), and of PAI-1 and PAI-2 in 51 pairs of cytosols (43 melanomas, 8 nevi) (Table 2). The mean uPA and PAI-1 concentrations in melanomas were significantly higher than in normal skin ($p < 0.0001$). The mean uPA concentration was higher in melanomas than in dysplastic nevi and higher in dysplastic nevi than in normal skin, however statistically insignificant ($p > 0.05$). Mean PAI-1 concentration was higher in melanomas than in dysplastic nevi, and higher in dysplastic nevi than in normal skin with significant results ($p = 0.02$). Mean PAI-2 concentration was not significantly higher in normal skin than in dysplastic nevi and melanomas ($p > 0.05$). There was a significant positive correlation ($r_s = 0.45$) between melanoma uPA and PAI-1 concentrations. Significant correlations between melanoma and normal skin concentrations for uPA, PAI-1 and PAI-2 were found ($r_s = 0.73; 0.54; 0.38$) (Figure 1).

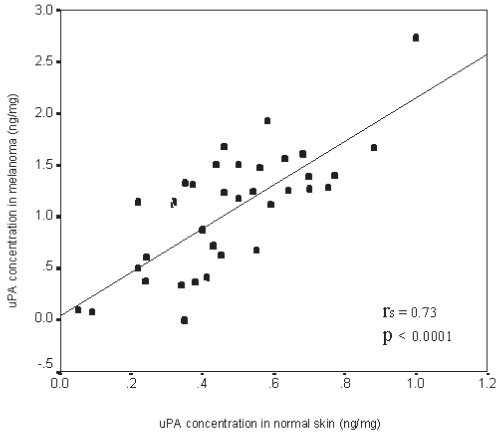

Figure 1. Correlation between melanoma and normal skin uPA concentrations in ng/mg proteins (n = 36, $r_s = 0,73$, $p < 0,0001$).

Table 2. Differences of uPA, PAI-1 and PAI-2 concentrations between melanomas, nevi and normal skin

	uPA (ng/mgp)	PAI-1 (ng/mgp)	PAI-2 (ng/mgp)
Melanoma	1,08 ± 0,58	14,07 ± 16,55***	9,21 ± 18,32
Dysplastic nevi	0,99 ± 0,58	4,84 ± 8,32****	13,44 ± 17,32
Normal skin around Melanoma	0,48 ± 0,21*	2.07 ± 1,83**	13,05 ± 19,72
Normal skin around Dysplastic nevi	0,51 ± 0,36	0,89 ± 0,91	28,8 ± 39,55

Levels are mean ± sd

*, ** $p < 0,0001$, statistically significant lower uPA and PAI-1 normal skin levels according to melanoma levels

***, **** $p = 0,021$ statistically significant higher PAI-1 melanoma levels according to nevi and higher nevi levels according to normal skin levels

Correlation between melanoma uPA, PAI-1 and PAI-2 concentrations and relevant prognostic factors

Melanoma uPA, PAI-1 and PAI-2 concentrations were compared to established histomorphological factors (Table 3). In contrast to uPA and PAI-2, PAI-1 concentrations were significantly lower in melanomas of Breslow tumour thickness ≤ 0.75 mm, Clark level of invasion 0+I, without microscopic ulceration on the tumour surface and absents vascular invasion of the tumours.

Much higher positive correlations (r_s = 0.67, 0.62 and 0.63 respectively; $p \leq 0.02$) between u-PA and PAI-1 concentrations were found in the group of the melanomas of Breslow thickness > 0.75 mm, with the level of Clark invasion III and present vascular invasion.

Discussion

In the present study using specific ELISA performed on tumour extracts of primary melanomas less than 1.5 mm thick, higher uPA and PAI-1 and lower PAI-2 concentrations than in nevi and normal skin were found. The correlation between uPA, PAI-1 and PAI-2 concentrations in melanomas and normal skin was found to be positive.

The prognostic value of uPA and PAI-1 in early stages primary malignant melanoma has not yet been investigated. However, a strong correlation between the metastases of the human melanoma cells in the nude mouse model as well as in the human cutaneous melanoma and expression of uPA and PAI-1 was found.[6] Dysplastic nevi are supposed to be an important risk factor for the development of the cutaneous melanoma. In 1979 Fräki et al. found out higher concentrations of plasminogen activators in primary melanomas and melanoma metastases comparing to extracts of nevi.[11] Recently De Vries et al reported that uPA, PAI-1, PAI-2 and uPAR appeared frequently in advanced primary cutaneous and uveal melanoma and melanoma metastasis lesions.[7,8,12] However uPA and PAI-1 accumulation was observed also in atypical nevocytes, whereas uPA proteolytic activity was detected only in melanomas.[13]

Table 3. Differences of mean concentrations of uPA, PAI-1 and PAI-2 in primary cutaneous melanomas with histomorphological prognostic factors

Variable	N	uPA±SD (ng/mgp)	p[a]	N	PAI-1±SD (ng/mgp)	p[a]	N	PAI-2±SD (ng/mgp)	p[a]
Breslow									
≤ 0,75 mm	22	1,01±0,54	n.s.	28	9,68±11,92		28	10,89±22,34	n.s.
> 0,75 mm	14	1,2±0,65		15	22,25±20,92	0,016	15	6,07±5,35	
Clark									
0+I	9	0,89±0,55		12	4,74±6,45		12	15,97±32,36	
II+III+IV	27	1,15±0,59	n.s.	31	17,67±17,89	0,001	31	6,59±7,78	n.s.
Ulceration									
Yes	7	1,38±0,74		7	27,76±23,27		7	3,2±2,68	n.s.
No	29	1,01±0,53	n.s.	36	11,41±13,82	0,015	36	10,38±19,82	
Vascular invasion									
Yes	2	1,99±1,04	n.s.	3	37,77±23,01		3	5,13 ±6,74	n.s.
No	15	1,02±0,55		20	9,5±12,74		20	11,48±25,62	
Undetermined	19	1,04±0,52		20	15,1±16,6	0,017	20	7,55±8,33	

[a]Analysis of variance, two-tailed t-test, $p \leq 0,05$; n.s. = not significant

Previously, uPA and PAI-1 have been claimed to be of independent prognostic value for disease free and overall survival in breast cancer patients.[14,15] In addition to breast cancer, components of plasminogen activation system also have a prognostic value in colorectal, gastric, oesophageal, bladder, endometrian and ovarian cancer.[2,5] Similarly Nekarda et al have found a stronger prognostic impact of PAI-1 than that of uPA in completely resected gastric cancer.[16]

The presence of the components of the PAs in malignant melanoma has earlier been studied using immunohistochemistry (IHC), in situ zymography and in situ hybridisation. Both, ELISA and IHC have their specific advantages. ELISA methods give an objective quantification of analyte levels, whereas IHC yields at best semi-quantitative information. ELISA and IHC may detect fractions of PA components with different efficiencies.[17,18] At present, ELISAs measuring the levels of uPA and PAI-1 performed consistently well, at least by their more extensively proven clinical value and unequivocal interpretation as demonstrated by the Quality Assurance Center in Nijmegen and from the European Organisation for Research and Treatment of Cancer (EORTC).[10]

Breslow tumour thickness, Clark level of invasion, ulceration and vascular invasion are the most important histological variables predicting melanoma outcome. The prognostic role of other possible markers like molecular and biochemical is not known yet. Our results prove the presence of the correlation of uPA and PAI-1 concentrations with prognostic value of Breslow thickness, Clark invasion, ulceration and vascular invasion also in early melanoma stage I. We observed that higher PAI-1 concentrations were associated with melanomas of Breslow > 0.75 mm, Clark II+III+IV, present ulceration and vascular invasion, which are prognostic adversely. Significant positive correlation between uPA and PAI-1 in melanomas with poor prognosis proves that also uPA has a prognostic impact. It indicates that their role in melanoma growth is co-dependent.

There are various speculative explanations of the role of excessive PAI -1 production in the tumour:[14,16,19] a) PAI-1 is important for reimplantation of circulating tumour cells at distant loci; b) since PAI-1 is present in endothelial cells and platelets, increased PAI-1 levels may reflect a high degree of angiogenesis, thus favouring tumour spread and metastasis; c) PAI-1 binds to adhesive glycoprotein vitronectin and influences cell adhesion and migration.

The prognostic role of PAI-2 is not well known yet. In breast cancer patients with high uPA concentrations, PAI-2 correlated with good prognosis.[20] In colorectal cancer, however, high PAI-2 concentrations were associated with aggressive disease.[21]

Our results prove that using ELISA it is possible to quantify the uPA, PAI-1 and PAI-2 concentration in very small specimens of early melanoma lesions.

Analogous to breast cancer, uPA and PAI-1 could become an additional prognostic factor for the progression of melanoma next to the established histological criteria. Furthermore, the fundamental role of uPA and PAI-1 in tumour invasion and metastasising indicates that these factors should be explored as targets for tumour biology - oriented therapies.[22]

To conclude, our results indicate that using ELISA uPA, PAI-1 and PAI-2 concentrations can be measured also in small samples of primary melanoma and that both uPA and PAI-1 might also be of prognostic importance in primary malignant melanoma. These findings need to be confirmed in further studies where relationship between uPA, PAI-1 and patient survival will be investigated.

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