Histol. Histopathol.

Poly(ADP-ribose) in the skin and in melanomas

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Summary

Cutaneous melanoma (CM) and uveal melanoma (UM) represent the most aggressive pigment cell tumor types. Our investigation examined the signaling molecule poly(ADP-ribose) (PAR) in CM and UM. We have demonstrated PAR in keratinocytes, sebocytes, hair follicles, endothelial cells and in subcutaneous adipocytes in the normal skin indicating that PAR may regulate physiological functions in these cell types. Furthermore, CM cells were PAR positive and tumor invasion level/thickness of CM correlated with the PAR content of the cell nuclei, with higher Clark and Breslow indices and AJCC scores associating with higher PAR content. This correlation was especially marked in the samples of female patients. In UM tumors (n=12) a slight overall and strong perivascular PAR staining was observed with considerable individual variations. In view of recent successful clinical trials with PARP inhibitors as adjuvant chemotherapeutic agents, our results suggest that melanomas may display differential sensitivity towards this novel therapeutic modality which should be considered for the selection of patients.

Introduction

Cutaneous melanoma (CM) is the most aggressive form of skin cancer among the Caucasian population. It is the sixth most common cancer and its incidence is on the rise in western populations. In the United States an estimated 68,720 new cases (39,080 men and 29,640 women) were reported in 2009 causing 8650 deaths (5550 men and 3100 women). The number of total body acquired melanocytic nevi and the occurrence of previous epithelial skin cancer represent major risk factors for the development of CM (Lawson *et al.*, 1994; Marghoob *et al.*, 1996; Jackson *et al.*, 2000; Bakos *et al.*, 2002). Solar damage is the major environmental causal factor in all skin cancers, and intermittent intense exposures to sunlight and/or severe sunburn especially during childhood (Hedges & Scriven, 2008) are also important environmental risk factors for CM.

The prognosis of melanoma depends on the stage at which it is detected as patients who are diagnosed early and treated surgically with excision of the tumors have the highest chance for a complete cure. Unresectable or advanced metastatic diseases, however, have a poor prognosis (Garbe *et al.*, 1995; Ahmed 1997). Therapeutic strategies to combat CM include chemotherapy, bio-chemotherapy, immune adjuvants, cancer-specific vaccines, cytokines, monoclonal antibodies and specific immunostimulants (Guerry & Schuchter, 1992, Schadendorf 2002).

Another pigment cell-derived malignancy, uveal melanoma is the most common adult primary intraocular tumor (Sato *et al.*, 2008) however it appears to be unlinked to photodamage (Singh *et al.*, 2004). Uveal melanoma can be classified as spindle-A, spindle-B, mixed-type and epitheloid melanomas with the order of degradation of the prognosis marked by increasing proportion of mitotic cells, metastatic capacity and extraocular extension. Possible treatments range from local laser photocoagulation, radiation therapy to enucleation dependent on the size of the melanoma.

Inhibition of poly(ADP-ribose) polymerases (PARPs) recently emerged as a novel therapeutic modality in the treatment of certain types of cancer. From the 17 member PARP enzyme family, the nuclear enzymes PARP-1 and PARP-2 are DNA nick sensors (De Murcia & Menissier de Murcia, 1994; Ame *et al.*, 2004,). Their binding to damaged DNA induces enzyme activation whereby PARPs cleave NAD^+ to nicotinamide and ADP-ribose and polymerize the latter to form branched poly(ADP-ribose) (PAR) polymer (Schreiber *et al.*, 2006). PARylation regulates many processes including chromatin organization, replication, transcription, metabolism and cell death (Virag & Szabo, 2002; Erdelyi *et al.*, 2005). PAR synthesis is involved in the initiation of the DNA damage response facilitating DNA base excision repair (Schreiber *et al.*, 2006; Hassa & Hottiger, 2008). PARP inhibitors were proposed to be useful as adjuvant therapeutic agents in cancer patients including melanoma patients receiving chemotherapy or irradiation (Tentori *et al.*, 2003; Kasper *et al.*, 2007; Plummer *et al.*, 2008; Chalmers, 2009).

The expression of PARP-1 has been investigated in CM (Staibano *et al.*, 2005), however the signaling molecule PAR itself was not detected. PARP-1 is usually expressed abundantly in most cell types and its function is regulated primarily at the level of activity. PARP activity is affected by DNA breaks, special DNA structures, phosphorylation and acetylation. Thus PAR levels do not necessarily correlate with protein level of PARP-1 or other PARP enzymes. Here we set out to investigate the differences in PAR content in various types of cutaneous and uveal melanomas.

Materials and methods

Tissue samples

Studies involving human tissue samples were conducted in accordance with the declaration of Helsinki and were approved by the Ethical Committees of the Medical and

Health Science Center of the University of Debrecen and Semmelweis University (for cutaneous and uveal melanoma samples, respectively).

Retrospective analysis was performed on formalin-fixed, paraffin embedded routine histology sections from the archive of the Department of Dermatology (University of Debrecen, Hungary). Melanoma patients (age 10-92 years) treated between 2002-2007 were selected. Clark stage I-V primary melanomas (Breslow 0,06-11,00 mm, T1a-4b) and cutaneous melanoma metastases were analyzed. Specimens were classified by histology as superficial spreading melanoma (17 subjects), lentigo melanoma (17 subjects), nodular melanoma (17 subjects), skin metastasis (15 subjects). Healthy skin (around melanocytic naevi) and melanocytic naevi were used as controls.

Uveal melanoma samples used in this study were from patients undergoing enucleation due to UM at the Department of Ophthalmology (Semmelweis University, Budapest, Hungary) between 2005 and 2006. We analyzed 12 formalin fixed, paraffin embedded tumors (n=12; 5 spindle, 3 epitheloid and 4 mixed).

Materials

The anti-poly(ADP-ribose) antibody (clone 10H) was purchased from Alexis Biochemicals, (Lausanne, Switzerland) (Erdelyi *et al.*, 2009). The Vector Elite ABC kit (*Vector Laboratories*, Burlingame, CA, USA) was used for the immunostaining procedure. Unless stated otherwise, all materials were obtained from Sigma-Aldrich.

Immunohistochemistry

The immunohistochemical localization of PAR was performed as follows. After deparafinization, 5 μm sections were treated with H_2O_2 [3% (v/v) in methanol] for 20 min in order to block endogenous peroxidase activity. After washing in PBS for 5 min, sections were

subjected to antigen retrieval by heating for 5 min in a pressure cooker in sodium citrate buffer (0.01 M, pH 6.0). Sections were rinsed in distilled water and washed for 5 min in PBS followed by incubation with a mixture of 1% (w/v) bovine serum albumin and 1 % (v/v) horse serum at room temperature for 20 min, to prevent non-specific reactions. Subsequently, sections were incubated overnight at 4°C with anti-PAR monoclonal antibody diluted in blocking solution (1% BSA, horse serum). After washing with PBS (3x10 min), sections were further incubated for 45 min at room temperature with biotinylated horse anti-mouse IgG secondary antibodies (provided in the Vector kit and used at 1:600 dilution in blocking solution). The sections were then washed (3 × 10 min) with PBS, and treated with 2% avidin–biotin–peroxidase complex (ABC) reagent for 30 min. Afterwards, sections were washed with PBS (3×10 min) and reacted with Ni-DAB substrate (1,6 mM 3,3′-diaminobenzidine tetrachloride, 140 mM NaCl, 90 mM $NiSO_4$, 100 mM Na-acetate, 3 mM H_2O_2, pH 6.6) for 4 minutes. After rinsing sections in 0.1M TBS (pH 7,2), the color was enhanced by incubating the sections for 3 min in 0.5% cobalt chloride (in 0.1 M TBS, pH 7,2). After rinsing in distilled water, sections were counterstained with Chromotrop 2R (*Chroma*, Stuttgart, Germany) solution (500 mg/l Chromotrop 2R, 0.005% (v/v) acetic acid). Negative immunohistochemical controls (isotype control antibody) were included in each staining run. Initially, PAR detection was performed after removal of melanin from the sections but the presence of melanin did not appear to interfere with the immunodetection, therefore this was omitted from the final procedure.

Scoring and statistical analysis

Nuclear staining was scored semiquantitatively by two experienced independent researchers on an arbitrary scale as follows: 0 (negative), 1 (low), 2 (moderate), 3 (partly low

to moderate, partly strong), 4 (strong). Immunostained slides were compared to H&E stained counterparts in order to ensure that melanocytes were scored.

PAR staining scores of the two independent investigators were averaged and then correlated with Breslow index, Clark stage and AJCC scores using Graphpad and SPSS17 softwares. The Spearman's coefficient of correlation was calculated and its significance was determined at $p<0.05$.

Results

PAR formation in healthy skin and CM

In normal skin, PAR formation was observed in various areas (Figure 1, A-G). Sebocytes and hair follicle cells, epidermal keratinocytes, endothelial cells and subcutaneous adipocytes all demonstrated positivity for PAR.

In cutaneous melanoma sections we observed nuclear PAR staining in the tumor cells. However, the intensity of the staining was highly variable (Figure 1, H, I, J and Table 1). The staining intensity correlated with Breslow index, Clark staging and AJCC scores of the melanomas (Figure 2). The Spearman's rank coefficients for the correlation between the PAR signal and Breslow index, Clark stage and AJCC scores for all samples were r=0.4125 (p=0.0025), r= 0.3257 (p=0.0197) and r=0.3657 (p=0.0083), respectively. Interestingly, this correlation was more pronounced in the case of female patients: Spearman's rank correlation coefficients and their statistical significance values for the female samples were (in the same order as above) r=0.6678 (p=0.0004), r=0.5584 (p=0.0046), r=0,5486 (p=0.0055) and for the male patients the values were r=0.3227 (p= 0.1006), r=0.2283 (p=0.2520) and r=0.3575 (p=0.067).

Specificity of the PAR staining was further confirmed by staining WM35 human melanoma cells (Fig 3A), that had been treated with H_2O_2 (5 min, 400 μM) in the absence (Fig 3B) or presence of the PARP inhibitor PJ34 (10 μM) (Fig 3C).

PAR formation in uveal melanoma

In the unaffected (normal) area of the eye, nuclear PAR staining was detected in the nuclear layers of the retina (Fig 4) and around blood vessels in the choroid (A). By comparison, PAR staining varied in location within the tumors (Fig 4B and C). In some samples, staining was most intense around the blood vessels (Fig 4D). Although we observed clear differences in PAR staining between individual tumors, we were unable to correlate PAR immunopositivity with the grade of the tumors due to the low number of cases in the study.

Discussion

Our present study provides strong evidence that poly(ADP-ribose) is synthesized in the normal human skin and can also be detected in CM and UM samples.

In normal skin, PAR positive cells were found in the epidermis. Keratinocytes that represent the majority of cells in the epidermis have already been described as expressing PARP-1 (Ross *et al.*, 1998; Szabo *et al.*, 2001). Moreover, several groups have reported a dominant expression of PARP-1 in cultured primary keratinocytes or HaCaT cells (Malanga & Althaus, 1994; Szabo *et al.*, 2001; Bakondi *et al.*, 2002; Pachernik *et al.*, 2002; Bakondi *et al.*, 2003; Bakondi *et al.*, 2004; Cals-Grierson & Ormerod, 2004). In keratinocytes, PARP-1 activation has been linked to inflammatory skin diseases and sunburn (Szabo *et al.*, 2001; Farkas *et al.*, 2002; Bakondi *et al.*, 2004; Bai *et al.*, 2009). PARP1 has also been shown to regulate the expression of proinflammatory cytokines and chemokines (IL-1, TNFa, MIP-1a,

MIP-2, MCP-1, etc.) (Szabo *et al.*, 2001; Bakondi *et al.*, 2003; Cals-Grierson & Ormerod, 2004,; Bai *et al.*, 2009) and oxidative stress–induced cell death (Bakondi *et al.*, 2003; Bakondi *et al.*, 2004). Our current study indicates that PAR may also serve as a signaling molecule in keratinocytes as the polymer could be detected in keratinocytes in the healthy skin. However, whether PAR synthesis in keratinocytes is induced by DNA breaks (e.g. generated by topoisomerases) or by DNA break-independent processes, requires further investigation. Intense PAR staining was found in hair follicle cells. This may be due to the rapid cycling of cells as PARylation has been implicated in the regulation of proliferation (Virag & Szabo, 2002).

We also detected PAR polymer in sebocytes and adipocytes suggesting a new role for PAR in these lipid accumulating cell types. The role of PARP-1 in adipocytes has already been proposed (Janssen & Hilz, 1989; Hsu & Yen, 2006), however, its presence in mature adipocytes has not yet been reported. PARP-1 has been shown to interact with the retinoid X-receptor (RXR) in the RXR – peroxisome proliferator activated receptor γ (PPARγ) nuclear receptor heterodimer (Miyamoto *et al.*, 1999) that controls sebocyte lipid accumulation during sebocyte differentiation (Rosenfield *et al.*, 1999; Kim *et al.*, 2001). Recent data suggests that recruitment of PARP-1 to active nuclear receptors is initiated by DNA strand breaks during receptor activation (Ju *et al.*, 2006) thus possibly accounting for the presence of PAR in these cells.

PARP-1 expression has previously been detected in human melanomas with correlation established between the intensity of expression and the clinical stage of the tumor (Staibano *et al.*, 2005). However, PAR content may reflect the intensity of cellular PAR metabolism better than PARP-1 expression. Therefore, our findings that PAR content correlates with the Breslow index, Clark stage and AJCC score of the tumor may have therapeutic implications in patients treated with PARP inhibitors as adjuvant chemotherapeutic agents.

The question is what may cause PAR accumulation in melanoma cells. Melanoma cells have a higher cell division rate than the normal surrounding tissue. We have previously reported increased PARP activity in mitotic cells (Bakondi *et al.*, 2002) which may help explain PAR synthesis in melanoma cells. However, the intensity of PAR staining was rather uniform in the CM specimens even though most cells are obviously not in mitosis suggesting that PAR synthesis may not be mitosis-related in melanoma cells. However, increased oxidative stress has also been reported in both CM (Sander *et al.*, 2003) and UM (Blasi *et al.*, 1999) leading to an increased risk of DNA damage that in turn may lead to PARP activation. PAR synthesis may also be related to tumor oxygenation as indicated by intense PAR staining around the tumor blood vessels in UM sections. In tumor tissues, *de novo* angiogenesis is important for providing nutrient and oxygen supply for tumors. Angiogenesis requires the concerted expression of VEGF and HIF-1 with the transcription of both of these factors requiring PARP-1 (Obrosova *et al.*, 2004; Martin-Oliva *et al.*, 2006,).

Our finding that the level of correlation between PAR levels and clinical scores was more pronounced in females than in males raises new questions. Gender has been reported to be an etiological and prognostic factor in melanoma and was found to affect various other skin diseases as well (Dao & Kazin 2007). Furthermore, gender differences in the role of PARylation have previously been reported in animal models of stroke and endotoxin shock (Hagberg et al. 2004; McCullough 2005; Mabley et al., 2005) and were proposed to be due to the protective effect of estrogen and to distinct sex-based cell death programs (Mabley et al., 2005; Szabó et al 2006; Yuan et al. 2009). As several questions including the pathways leading to PAR production in melanoma and the role of PARylation in melanoma cell death are not precisely understood, the implications and significance of this finding requires further investigation.

Since inhibition of PARP-1 impairs base excision repair, targeting PAR metabolism has become a therapeutic opportunity in the treatment of CM (Tentori *et al.*, 2005; Plummer *et al.*, 2008; Fong *et al.*, 2009). Adjuvant chemotherapy using PARP inhibitors (e.g. in combination with temozolomide) for the treatment of solid tumors is currently in different phases of clinical trials (Fong *et al.*, 2009). Our results demonstrating individual differences in PAR content in patients with different stages of melanoma suggest that CM patients should be carefully selected for PARP inhibitor treatment.

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Legends to figures and tables

Fig. 1. PAR in the normal skin and melanocytic naevi

PAR was detected in epidermal keratinocytes (A), hair follicle cells (B), endothelial cells and cells of the vessel walls (C, D) sebocytes (E), subcutaneous adipocytes (F). Melanocytic naevi also contained PAR positive cells (G). Scale bars represent 50 μm (A, B, C, F, G) or 100 μm (D, E). PAR was also detected in CM samples. Panels H, I and J display representative images of PAR staining in SSM, lentigo melanoma and nodular melanoma, respectively. Negative control (isotype control) staining of a nodular melanoma section is shown on panel K.

Scale bar equals 40 μm (Panels (H, I, J, K).

Fig. 2. Statistical evaluation of the PAR staining

PAR staining scores were correlated with Clark, Breslow and AJCC scores. For the correlation of these indices and PAR staining, the Spearman's rank correlation coefficient was calculated which revealed a statistically significant correlation (p values are shown on the figure).

Fig. 3. PAR staining in cultured melanoma cells

In order to demonstrate the specificity of the PAR staining with the 10H antibody, we stained control (A), and H_2O_2-treated (5 min, 400 μM) (B, C) melanoma cells with the anti-PAR specific 10H antibody. Melanoma cells on panel C were also pretreated with the PARP inhibitor PJ34 (30 min, 10 μM).

Fig. 4. PAR staining in the retina and in uveal melanomas

PAR was detected in the nuclear layers of the retina (A; stars) and strong immunopositivity could be seen around blood vessels of the choroid (A; arrows). In UM tumors (B and C) PAR positivity varied in intensity with perivascular regions often showing more intense staining than other tumor areas (D).

Table 1. Summary of PAR expression in CM patients

CM cases are presented in groups of different histological classes (SSM: superficial spreading melanoma; LM: lentigo melanoma; NM nodular melanoma; MET metastasis).

Age, sex, Clark stage and Breslow index, AJCC scores and PAR staining scores are shown.

Table 2. Summary of PAR expression in UM patients

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Age	Sex	Breslow	Clark	AJCC	Histology	PAR
62	M	0,45	3	1	SSM	1
33	M	0,24	3	1	SSM	1
69	M	1,12	3	1	SSM	1,5
44	F	0,48	3	4	SSM	1
44	F	6	3	2	SSM	3
38	F	0,12	2	1	SSM	2
73	F	1,75	4	2	SSM	2
63	F	1,1	3	2	SSM	3
40	F	7	4	4	SSM	3
69	F	2	4	2	SSM	2
58	M	0,61	3	1	SSM	1
44	M	0,88	3	1	SSM	2
63	M	0,25	2	1	SSM	2
51	M	0,65	3	1	SSM	3
38	M	0,55	3	1	SSM	3
50	M	0,31	2	1	SSM	1
45	M	1,12	3	2	SSM	3,5
67	M	0,12	2	1	LM	1
71	M	0,06	2	1	LM	2
79	F	0,42	3	1	LM	1
66	F	0,2	1,5	1	LM	2
81	F	0,07	2	1	LM	2
80	F	0,18	2	1	LM	2
85	F	0,3	2	1	LM	2,5
73	F	0,22	3	1	LM	2
65	F	0,28	2	1	LM	1
45	F	0,2	1	1	LM	1
69	M	0	1	1	LM	3
82	M	0	1	1	LM	3
70	M	0,85	3	1	LM	4
77	M	1,4	3	2	LM	2
75	M	0,3	2	1	LM	2
71	M	0,43	2	1	LM	3
68	M	0,56	3	1	LM	4

Age	Sex	Breslow	Clark	AJCC	Histology	PAR
44	M	2,5	5	1,5	NM	1,5
53	M	4,5	4	2	NM	2
39	F	2	3	2	NM	2,5
68	F	6,5	4	4	NM	3
65	F	5,5	5	4	NM	3
42	F	2	4	2	NM	3
51	F	3	2,5	3	NM	2
51	F	2,5	4	3	NM	2
64	F	2,45	3	3	NM	3
75	F	11	5	4	NM	4
35	F	8,5	5	4	NM	2,5
55	M	0,65	3	1	NM	1,5
69	M	1,61	4	2	NM	3,5
40	M	4	4	3	NM	3
66	M	3,8	4	3	NM	4
56	M	1	3	1	NM	4
44	M	2,25	4	3	NM	4
83	M				MET	4
66	M				MET	3
10	M				MET	2,5
83	M				MET	3
92	F				MET	3
45	F				MET	2
46	F				MET	2
60	F				MET	4
64	F				MET	3,5
10	F				MET	2
52	M				MET	3
62	M				MET	4
68	M				MET	3
77	M				MET	3,5
51	M				MET	2

Table 1.

Age	Histopathologic cell type	Extraocular extension	PAR staining
64	spindle	no	1
55	mixed	no	2
55	epitheloid	scleral infiltration	2
71	epitheloid	opticus infiltration	2
76	spindle	no	4
46	spindle	no	4
53	mixed	scleral infiltration	3
46	mixed	vortex vein	3
39	spindle	no	3
81	spindle	scleral infiltration	3
56	epitheloid	no	3
54	mixed	no	2

Table 2.