SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Role of oxidative stress and poly(ADP-ribose) in melanomas and proliferative diabetic retinopathy

by
Dr. Zsuzsanna Géhl

Supervisor: Prof. Dr. László Virág

UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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By Zsuzsanna Géhl

Supervisor: Prof. László Virág, DSc

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Examination Committee**:	Prof. János Szöllősi, DSc
Members of the Examination Committee:	Tamás Csont, PhD
	István Bak, PhD

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Head of the **Defense Committee**: Prof. János Szöllősi, DSc

Reviewers:	István Lekli, PhD
	Péter Vámosi, PhD

Members of the Defense Committee:	István Bak, PhD
	Tamás Csont, PhD

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INTRODUCTION

Oxidative stress is involved in the pathogenesis of most diseases, and the consequential activation of poly(ADP-ribose) polymerase (PARP), a member of DNA damage sensing and repair system also contributes to a variety of pathological processes. In our study, we investigated the relationship between the PAR (poly(ADP-ribose) status and clinical stage of melanomas. We also characterized the redox dysregulation in the vitreous humor in diabetic retinopathy.

1. The PAR and PARP inhibitors in oncology.

Cutaneous malignant melanoma (CM) is the most malignant form of skin cancer. There are several types of CM, superficial spreading melanoma (SSM) being the most common type, nodular melanoma (NM) and lentigo maligna (LM) are also relatively common. The primary treatment for melanoma is surgical resection, in cases of metastasis chemotherapy or radiotherapy are used. **Uveal melanoma** (UM) is the most common primary intraocular malignancy in adults. Survival rates are usually low, due to the high incidence of metastases. As the tumor may not cause symptoms in the early stages, conservative treatment is not feasible because of late diagnosis and large tumor size, most often the preferred surgery is enucleation (removal of the eyeball).

Cutaneous and uveal melanomas differ from each other in their progression due to their different genetic background and metastatic pathway. Their different behavior can be explained by the ocular immune privilege, a characteristic retained by metastasized uveal melanoma cells. Many drugs used effectively for cutaneous melanoma are inefficient in the therapy of primary and metastatic uveal melanoma. BRAF and MEK inhibitors are beneficial in advanced metastatic BRAF mutant CM, but chemotherapy of UM leaves much to be desired. Unfortunately, no medication appears to improve survival rates, chemotherapeutic strategies used in oncology are ineffective in UM. The molecular pathways of the biological behavior of UM must be further investigated in order to develop successful drug treatment like in other cancer types.

Reactive oxygen and nitrogen species (ROS/RNS) are associated with the pathogenesis of several diseases. A late process in ROS/RNS-associated tissue damage is the

increase of PAR metabolism which allows time for clinical treatment (wide therapeutic window). PAR polymer is synthesized by PARP enzymes which cleave NAD^+ and synthesize polymers by attaching the ADP-ribose units. This posttranslational protein modification regulates several cellular processes including cell death, DNA repair, replication, transcription and differentiation. PARP activation also plays an important role in the pathological processes of the eye. The best-known function of PARP is maintenance of genome integrity. PARP inhibition impairs base excision repair (BER). Based on the central role of poly-ADP-ribosylation in DNA damage repair, PARP inhibition would be expected to increase the rate of mutation and tumor development. However, experimental studies provide evidence that PARP inhibitors reduce tumor development and metastasis.

Pharmacological inhibition of PARP proved to be effective in the therapy of tumors with HRR (homologous recombination repair) defects caused by BRCA mutations. Thus, PARP inhibitors are clinically approved drugs in BRCA-related ovarian cancer. They act through synthetic lethality. While HRR repairs DNA damage in normal cells, the defect of homologous recombination together with the inhibition of non-homologous DNA repair prove lethal in tumor cells. PARP inhibitors represent a new class of promising chemotherapeutic agents, but their efficiency in melanomas needs further investigation. PARP inhibitors show synthetic lethality with several protooncogenes. They entered clinical trials in uveal melanoma with germline mutation of BAP1, while they were effective as adjuvant chemotherapy in combination with temozolomide in metastatic cutaneous melanoma. Study of PARPs in melanomas might contribute to the success of clinical studies and help to design further clinical trials. Determining PAR levels in tumors allows the selection of patients that could benefit from PARP inhibitor treatment.

2. Oxidative stress in the development of diabetic retinopathy (DRP)

Oxidative stress plays a key role in several pathological processes like hyperglycemia and diabetes, including the development of diabetic complications like DPR. Free radicals are produced during normal metabolism of carbohydrates and eliminated by the antioxidant system of the body. Elevated blood glucose levels cause an imbalance between free radicals and antioxidants and result in oxidative stress. **AGEs (advanced glycation end products)** play a critical role in the process. AGEs are formed in a non-enzymatic glycation reaction of proteins or lipids with reducing sugars e.g. glucose. AGE formation is recognized as a major

factor in the development of diabetic complications, especially vascular complications. In the process of AGE formation, excess glucose reacts with a free amino group of amino acids, forming a non-stable Schiff-base that rearranges to form a more stable Amadori product. AGEs are formed by further reactions during weeks or months. AGEs and its precursors crosslink with proteins of the extracellular matrix, basal membrane and vascular wall components, altering their function. AGEs may bind to their cell surface receptor RAGE (receptor for advanced glycation end products), initiating proinflammatory and prooxidant pathways which have an impact in the development of retinopathy. The efficiency of glycemic control in diabetic patients is mirrored by serum (and tissue) levels of AGEs. In clinical practice, levels of glycated proteins, e.g. glycated hemoglobin A1c is widely used as a marker of glycemic control. Glycated HbA1c values reflect the blood glucose levels of the previous 6-8 weeks. Direct detection of reactive oxygen species is quite difficult; however, detection of relatively stable products of oxidative stress provides important information about the oxidant status of the organ. **Protein carbonyl** groups, being relatively stable and early products, are one of the best biomarkers of protein oxidation. A major component of the antioxidant system is **reduced glutathione (GSH)**, which is produced from the oxidized GSSG form in a reaction catalyzed by glutathione reductase. Tissue GSH levels provide important information about redox status.

AIMS OF THE STUDY

A growing body of evidence suggests the role of PAR in metabolic diseases and cancer. However, few studies investigated biomarkers of eye diseases in human tissues and samples. The contribution of oxidative stress in the development of diabetes and DPR is well established. Poor glycemic control, a major etiological factor in proliferative diabetic retinopathy (PDR), induces ROS production and oxidative stress, which contributes to diabetes-associated ocular complications. Besides proper control of carbohydrate metabolism, patients with severe PDR may require surgical intervention. During vitrectomy, the vitreous that fills the eye is surgically removed. The vitreous humor is a reservoir of carbohydrate metabolites, its chemical composition reflects the biochemical processes of the retina.

Aims of the study:

1. To detect PAR accumulation in melanoma cell lines.
2. To determine the PAR content in malignant cutaneous melanomas and its relation with the histological stages, with the severity of the CM, and to analyze gender differences in PAR content of cutaneous melanoma samples.
3. To study tissue sections from surgically removed eyes due to malignant uveal melanoma and investigate whether PAR content is related to tumor development like in several other cancer types.
4. To determine PAR levels in tumor-free eye tissues.
5. To determine oxidative stress markers and parameters of redox homeostasis in the vitreous in PDR patients: AGEs, which are involved in the pathogenesis of PDR; protein carbonyl, a marker of protein oxidation; and glutathione, a member of the antioxidant defence system.

MATERIALS AND METHODS

1. Immunohistochemical detection of PAR in cutaneous and uveal melanoma tissue sections

A retrospective analysis was performed on formalin-fixed, paraffin-embedded routine histology sections from the Department of Dermatology, University of Debrecen. **Cutaneous** melanoma patients treated between 2002 and 2007 were selected. Clark stage I-V primary melanomas (Breslow 0,06-11,00 mm, T1a-4b) and cutaneous melanoma metastases were analyzed. The study included samples from 17 superficial spreading melanomas (SSM), 17 lentigo melanomas (LM), 17 nodular melanomas (NM) and 15 skin metastases. Melanocytic naevi and healthy skin around melanocytic naevi were used as controls.

Uveal melanoma samples were formalin-fixed, paraffin-embedded sections from patients undergoing enucleation due to UM at the Department of Ophthalmology of Semmelweis University between 2005 and 2006. Embedded tissue samples from the archive were analysed retrospectively. Immunohistochemical studies were performed on routine histological sections of 12 samples: 5 spindle, 3 epitheloid and 4 mixed cell uveal melanomas.

PAR staining in cultured melanoma cells

To test the specificity of PAR staining, WM35 human melanoma cell line was used. Cells were purchased from ATCC (Manassas, VA, USA) and cultured in MCDB 153 medium (Sigma-Aldrich) containing 10% FCS, 20% Leibovitz medium (Life Technologies), 5 µl / ml bovine insulin, 1, 68mM calcium chloride, penicillin (50U / mL) and streptomycin (50µg / mL). Cells were cultured in culture media with H2O2 for 5 minutes at 400 µM. Part of the cells were pre-treated with 10 µM PJ34 PARP inhibitor for 30 min. PARs immunostaining on cells-as described below-after metanol (100%, -20ºC, 5 minutes) and paraformaldehyde (4% solution in PBS, pH 7.4, 10 minutes at room temperature) fixation by deleting the deparaffining and antigen detection steps.

Detection of PAR

The anti-PAR antibody (clone 10H) was purchased from Alexis Biochemicals (Lausanne, Switzerland). Vector Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for immunostaining. Detection of PAR was performed by immunohistochemistry. After deparaffinization, 5 µm sections were treated with H_2O_2 [3% (v/v) in methanol] for 20 min to

block endogenous peroxidase activity. After washing in PBS for 5 min, sections were subjected to heat-induced antigen retrieval for 5 min in sodium citrate buffer (0.01 M, pH 6.0), then rinsed with distilled water and washed again in PBS for 5 min. Sections were incubated 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 and horse serum). Excess antibody was removed by 3x10 min washing in PBS, then sections were further incubated for 45 min at room temperature with biotinylated horse anti-mouse IgG secondary antibody (provide in the Vector kit and used in 1:600 dilution in blocking solution). Sections were washed with PBS (3x10 min) and incubated with 2 % avidin-biotin-peroxidase complex (ABC) reagent for 30 min in PBS. After washing with PBS (3x10 min), samples were reacted for 4 min 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). After rinsing the sections in 0.1 M TBS (pH 7.2), the color was enhanced by incubation for 3 min in 0.5 % cobalt chloride (in 0.1 M TBS, pH 7.2). Following a rinsing in distilled water, sections were counterstained with Chromotrop 2R (Chroma, Stuttgart, Germany) solution (500 mg/ml Chromotrop 2R, 0.005 % (v/v) acetic acid). Negative immunohistochemical controls (isotype control antibody) were included in each staining process. Initially, PAR detection was performed after removal of melanin from the sections, but melanin did not appear to interfere PAR immunodetection, therefore this step was omitted from the final procedure.

2. Detection of oxidative stress markers in the vitreous of patients with proliferative diabetic retinopathy (PDR)

Vitreous samples of diabetic patients undergoing surgery were analysed in the study. Undiluted vitreous samples were obtained from patients at the beginning of vitrectomy and were stored in eppendorf tubes at – 80 °C until use. The study population of 10 diabetic patients were compared to the control group of 8 patients operated with epiretinal membranes. Samples were analysed to detect markers that indicate oxidative stress-related biochemical processes in the eye.

2.a. Determination of Advanced Glycation End Products (AGEs)

Amount of glycated proteins was determined using the Oxi-Select Advanced Glycation End Product Competitive ELISA Kit (Cell Biolabs, Inc., San Diego, USA) following the manufacturer's instructions.

2.b. Detection of protein carbonylation

ROS and RNS can oxidate amino acid side chains of proteins to carbonyl. For protein carbonyl determination, each sample was diluted with distilled water to reach a concentration of 20 µg protein in 200 µl. Then 50 µl of 80% trichloroacetic acid (TCA) solution was added, samples were vortexed and incubated on ice for 5 min. After centrifugation at 13,000 x *g* for 2 min, supernatants were removed and pellets were resuspended in 500 µl ice-cold acetone. Incubation for 5 min at -20 °C was followed by another centrifugation at 13,000 x *g* for 2 min. Acetone was removed and pellets were dissolved in 20 µl distilled water and used for protein carbonyl detection using OxiBlot Protein Oxidation Kit (Merck Millipore, Budapest, Hungary) following the manufacturer's instructions. After completing the derivatization step, the obtained dinitrophenol (DNP) product was quantified spectrophotometrically at 405 nm.

2.c. Glutathione assay

Samples were precipitated for 10 min with ice-cold 10 % TCA solution. After centrifugation (5000 x *g*, 4 °C) supernatant was used for further analysis. The glutathione assay was performed in 96-well plates. 1 M potassium phosphate buffer and 0.5 % o-phthalaldehyde were added to the samples and incubated for 30 min at room temperature, then fluorescence was measured at 390/460 nm. The mixture of supernatant and N-ethylmaleimide was used as a blank for each sample. A standard curve was prepared with GSH. Protein concentrations were determined with the BCA colorimetric method.

3. Statistical analysis

In **cutaneous and uveal melanoma** sections, nuclear staining was scored semiquantitatively by two independent, experienced 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 consider melanocyte staining. Scores of the two independent researchers were averaged and then correlated with Breslow index, Clark stage and AJCC scores using GraphPad and SPSS17 software. The Spearman's correlation coefficient was calculated, significance was determined at $p < 0.05$.

Concentration values of vitreous samples obtained from **diabetic and non-diabetic patients** were compared by Statistica 11.0 software using the Mann-Whitney U Test. The difference was considered significant if $p < 0.05$.

RESULTS

1. PAR formation in cutaneous and uveal melanoma

1.a. The specificity of PAR staining with 10H (anti-PAR) antibody was demonstrated in **human cultured melanoma cells**. Intensive PAR staining was observed in H_2O_2 treated cells compared to the control or PJ34 pretreated cells.

1.b. In **cutaneous melanoma** sections, we observed nuclear PAR staining in the tumor cells. Although the intensity of the staining was highly variable, staining intensity correlated with Breslow index, Clark staining and AJCC scores. Interestingly, this correlation was more pronounced in female patients. Spearmen's correlation coefficients and their statistical significance values for female patients were 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) és r=0,3575 (p=0,067). PAR levels correlate with the clinical stage of cutaneous melanoma, and this correlation is especially marked in female patients.

1.c. PAR accumulation was evaluated in **uveal melanomas** in the *unaffected* (tumor-free) areas of the eye, where PAR is normally formed. Nuclear PAR staining was detected in the nuclear layers of the retina and around blood vessels in the choroid.

PAR staining was variable *within the tumors*. In some samples, staining was intense around the blood vessels. Although there were clear differences in PAR staining between individual tumors, we were unable to correlate PAR immunopositivity with the tumor grade due to the low number of cases in the study.

2. Oxidative stress markers in the vitreous of proliferative diabetic retinopathy (PDR) patients

2.a. AGE levels in the vitreous

We set out to investigate whether increased glycation can be detected in the vitreous of diabetic patients, similarly to elevated levels of glycated hemoglobin in the serum. Increased AGE levels were found in the vitreous of PDR patients compared to control samples: 812.10

vs 491.69 ng/mg protein (p=0.058). This finding indicates that changes in glucose levels of the vitreous are similar to changes in the serum and other organs.

2.b. Oxidative stress in the vitreous

Oxidative stress is an imbalance between the production and elimination of ROS and RNS. If the elimination of ROS/RNS is less effective due to insufficient antioxidant capacity, protein oxidation and nitration, lipid and DNA damage may occur. The appearance of carbonyl groups on amino acid side chains is a sign of oxidative protein damage that can be triggered by a variety of ROS and RNS species. Protein carbonyl formation is an important biomarker of oxidative stress condition and contributes to cellular dysfunction in various diseases including diabetic cellular dysfunction. Protein carbonyl content in vitreous samples of DRP patients was determined. Protein carbonylation was significantly increased in the vitreous of DRP patients (2.08 vs 0.67 A_{405}/100 µg protein; p=0.017) compared to the control group. This finding indicates that oxidative stress is present in the vitreous humor and ROS/RNS can react with proteins in this compartment.

2.c. Antioxidant capacity of the vitreous

In addition to the production of ROS and RNS, the antioxidant repertoire is also a key determinant of the redox state of organs and tissues. Therefore, we analysed the antioxidant capacity in the vitreous of PDR patients. We determined the concentration of reduced glutathione (GSH), a major endogenous antioxidant. Levels of GSH were also significantly higher in diabetic vitreous samples as compared to control (4.53 vs 2.34 µmol/µg protein; p=0.019). (p<0.05).

CONCLUSIONS

1. PAR formation in cutaneous and uveal melanomas

1.a. Our results demonstrate that poly(ADP-ribose) is formed both in cutaneous melanoma (CM) and uveal melanoma (UM).

1.b. PARP-1 expression has previously been detected in human CM with correlation established between the intensity of the expression and the clinical stage of the tumor. However, our findings indicate that PAR content of different types of melanoma may reflect cellular PAR metabolism better than PARP-1 expression.

1.c. Our findings that PAR content correlates with 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 intensity of PAR staining was rather uniform in cutaneous melanoma samples, suggesting that PAR synthesis may not be mitosis-dependent in these tumors.

1.d. In CM samples the correlation between PAR staining and clinical scores was more pronounced in females. Gender has been shown to be an etiological factor in skin diseases including CM. Furthermore, gender differences in the role of PARylation have also been reported in several diseases.

1.e. PAR staining was detected in the nuclear layers of the retina and around blood vessels in the choroid of tumor-free eye samples.

1.f. Our study first described PAR formation in UM. Intense PAR staining was detected in blood vessel cells and in the tumor tissue around blood vessels in UM sections. These results suggest that PAR synthesis may be related to tumor oxygenation. *De novo* angiogenesis is important for providing nutrient and oxygen supply for the tumor.

Currently, PARP inhibitors are clinically approved drugs in oncological therapy. PARP inhibitors are promising agents for the treatment of tumors bearing BRCA mutation or defect in homologous recombination. Differences in cutaneous and uveal melanomas can be explained by their different protooncogene spectra. It might be important to reveal which tissue areas are possibly affected by PARP inhibitor treatment and it can be indicated by PAR levels. Since individual differences were found in PAR staining in patients with different stages of CM, patients should be carefully selected for PARP inhibitor treatment.

2. Determination of oxidative stress markers in diabetic retinopathy

PDR-related oxidative stress markers were determined in vitreous samples. Elevated AGE levels, a marker of protein glycation has been reported in diabetic patients. Even in case of close-to-ideal diabetes management, glycemic swings can increase protein glycation in the blood and various tissues. AGEs form in the eye in diabetes and contribute to diabetic complications.

2.a. Elevated AGE levels were found in the vitreous of PDR patients, which indicates higher glucose levels in the eye. Since metabolic exchange and equilibrium between systemic circulation and vitreous humor is considered to be slow, elevated AGE levels in the vitreous indicate sustained hyperglycemic periods and may contribute to the progression of diabetic retinopathy to the proliferative stage.

2.b. Tissue oxidative stress is typically accompanied by oxidative protein, DNA and lipid modifications. As a marker of protein oxidation, protein carbonyl content was measured in the vitreous. Elevated protein carbonyl levels in the vitreous of diabetic patients support the hypothesis that the vitreous humor is part of the diabetes-induced oxidative environment in the eye. The vitreous humor contains a high variety of proteins with albumin and type II collagen being the most notable ones. Thus, ROS/RNS may hit protein targets with high probability. Our results suggest that elevated protein carbonyl levels result from severe oxidative stress and contribute to diabetic complications. Thus, it raises the possibility of clinical applications of carbonylation inhibitors.

2.c. Obviously, the antioxidant system is activated in oxidative stress conditions. Considering the continuous presence of prooxidant stimuli, we expected lower antioxidant capacity in the vitreous of diabetic patients. In our study, we determined the concentration of reduced glutathione (GSH), a major endogenous antioxidant. We found higher GSH levels in the vitreous of PDR patients compared to control samples.This may indicate an adaptive response to increased oxidative stress, just like it has been reported for extracellular superoxide dismutase in PDR patients. Our study shows significant rearrangements in the redox homeostasis of human vitreous in diabetic patients with possible implications for the pathomechanism of PDR.

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List of publications related to the dissertation

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