Modulation of urokinase plasminogen activator system by poly(ADP-ribose)polymerase-1 inhibition

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Abstract The urokinase plasminogen activator (uPA) system is a complex regulator of extracellular proteolysis which is involved in various physiological and pathological processes. The major components of this system are the serine protease uPA, two inhibitors PAI-1 and PAI-2, and the receptor uPAR. It has been previously shown by several groups that the uPA system has an important role in cancer progression and therefore its possible prognostic and therapeutic value has been evaluated. The aim of this study is to tackle the role of poly(ADP-ribosylation) in the induction of uPA activity in a glioblastoma cell line, A1235. This cell line is sensitive to alkylation damage and is a model for drug treatment. The components of the uPA system and the level of DNA damage were analyzed after alkylation agent treatment in combination with poly(ADP-ribose)polymerase-1 (PARP-1) inhibition. Here we show that the increase in uPA activity results

from the net balance change between uPA and its inhibitor at mRNA level. Further, PARP-1 inhibition exerts its influence on uPA activity through DNA damage increase. Involvement of several signaling pathways, as well as cell specific regulation influencing the uPA system are discussed.

Keywords Urokinase plasminogen activator · Plasminogen activator inhibitor-1 · Poly(ADP-ribose)polymerase-1 · DNA damage

Abbreviations

uPA	Urokinase
PAI	Plasminogen activator inhibitor
uPAR	Urokinase receptor
PARP-1	Poly(ADP-ribose)polymerase-1
tPA	Tissue plasminogen activator
MNNG	N'-methyl-N'-nitro-N-nitrosoguanidine
3-ABA	3-Aminobenzamide
DMEM	Dulbecco's modified Eagle's medium
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
MGMT	O ⁶ -Methylguanine-DNA methyltransferase

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Introduction

Urokinase plasminogen activator system is a plastic system involved in cell migration and invasion

through extracellular matrix remodeling. The whole system consists of several elements. Urokinase type plasminogen activator (uPA) is a highly specific serine protease which is secreted in the form of the proenzyme. When activated, it converts plasminogen to a plasmin, a strong protease which can degrade extracellular matrix and activate latent collagenases. The cells also produce plasminogen activator inhibitors PAI-1 and PAI-2, to regulate uPA extracellular activity (Irigoyen et al. 1999). When uPA is bound to the cellular receptor (uPAR) it mediates cell surface dependent proteolysis, and is internalized and degraded in the cell if bound to its inhibitor (Dano et al. 1985; Conese and Blasi 1995; Aguirre Ghiso et al. 1999). uPA is tightly controlled at the level of transcription, mRNA degradation, protein production, localized activation and degradation. PAI-1 and PAI-2 also have complex regulation at the level of transcription and activation (Dano et al. 1985; Nagamine et al. 2005). uPA and PAI-1 have been involved in intracellular signaling through uPAR (Konakova et al. 1998). Besides its role in normal physiological processes, such as tissue remodeling, cell migration, and wound healing, the plasminogen activation system is associated with tumor invasiveness and metastasis and both, uPA and PAI, can be overexpressed in tumor cells or neighboring tissue (de Vries et al. 1996).

Poly(ADP-ribose)polymerase-1 (PARP-1) is an enzyme involved in a number of cellular processes. Its main role is in the signalization and recruitment of repair mechanism molecules on the site of DNA damage, especially to the site of single and double strand DNA breaks (Krishnakumar and Kraus 2010). It also has multiple roles in the regulation of apoptosis and necrosis. Further, PARP-1 can modify several transcription factors and modulate their function, taking part in this way in the processes of differentiation and gene regulation (Krishnakumar and Kraus 2010). We have previously shown that certain glioblastoma cell lines increase uPA activity upon alkylation damage (Brdar and Matulic 1988). Therefore, in this study we examined the involvement of PARP-1 in the induction of uPA activity in these cells. We found that PARP-1 inhibitor modulates uPA activity through its involvement in the process of DNA repair. Furthermore, we give evidence that uPA activity is a consequence of balance change between secreted uPA and PAI-1.

Materials and methods

Cell culture and treatment

Human glioblastoma cell line A1235, a kind gift from S. A. Aaronson (Giard et al. 1973), was cultivated in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, St. Louis, MO, USA), supplemented with 10 % fetal bovine serum (Gibco Life Technologies, Paisley, UK) at 37 °C and 5 % CO₂.

Cells were treated with N'-methyl N'-nitro N-nitrosoguanidine (MNNG), 3-aminobenzamide (3-ABA), PJ-34, JNK kinase inhibitor SP600125, Roscovitine, Wortmannin (all inhibitors from Sigma) and LiCl (Kemika, Zagreb, Croatia). All inhibitors were added to cell cultures 30 min before MNNG treatment and were present throughout the experiment. MNNG at indicated concentrations was applied 24 h before the endpoint. For caseinolysis, cells were incubated in DMEM without serum for additional 6 h and conditioned media were examined as previously described (Matulic and Brdar 2002).

A1235 cells were stably transfected with hPARP-EGFP plasmid (a kind gift from V. Schreiber (Hochegger et al. 2006)) and EGFP plasmid as a control (Clontech Laboratories, Madison, WI, USA), using Superfect Transfection Reagent (Qiagen, Venlo, Netherlands) according to the supplier's protocol. The clones were selected under G418 (Calbiochem, San Diego, CA, USA) and inspected under fluorescent microscope for green fluorescence (Olympus BX51, Tokyo, Japan).

Viability assay

Cells were plated at 10⁴ cells/well in a 96-well plate and treated with PARP-1 inhibitor, MNNG and their combination the following day. After 24 h the viability of the cells was assessed by means of tetrazolium colorimetric assay (XTT test, Biological Industries, Kibbutz Beit Haemek, Israel), according to the manufacturer's instructions. All samples were done in triplicate and analyzed statistically.

Cytotoxicity was analyzed by Trypan blue exclusion test (Fluka, Seelze, Germany). In brief, control and treated cells were collected and aliquots were mixed with an equal volume of 0.4 % Trypan blue. The percentage of nonviable cells that took up the dye was calculated by counting at least 200 cells per treatment on hemocytometer.

For growth curve, we treated the cells and counted them on daily intervals on Z2 Coulter Counter (Beckman Coulter, USA).

Senescence associated β -Gal assay

Cells were washed in PBS and fixed in 1 % glutaraldehyde in DMEM for 10 min, washed in PBS 2×5 min and stained for senescence associated β -galactosidase (SA- β -Gal) activity at pH 6.0 over 16–18 h as described previously (Dimri et al. 1995). The percentage of SA- β -Gal positive cells was estimated by counting under light microscope (Zeiss, Oberkochen, Germany).

Urokinase plasminogen activator activity

uPA activity was assayed by radial caseinolysis of conditioned, serum-depleted media, as described previously (Matulic and Brdar 2002). Namely, the amount of caseinolysis reflects the rate of plasmin activation, and the latter depends on the uPA activity in the conditioned medium. uPA activity in samples was estimated by interpolation from a calibration curve of human uPA (Leo Pharmaceutical Products, Denmark) and normalized according to protein concentration of corresponding lysates. Sample uPA activity was presented as a fold increase of control cell uPA activity. All samples were measured in quadruplicate and experiments were repeated at least twice.

Total RNA preparation, cDNA synthesis and RT-PCR

Total RNA was extracted from cells using Trizol (Invitrogen Co., Carlsbad, CA, USA), according to manufacturer's instructions. cDNA was synthesized from 4 μ g of total RNA by Superscript II Reverse Transcriptase (Invitrogen Co.), using oligo(dT) to prime first-strand synthesis, and cDNA was used for PCR reaction according to standard procedure (Sambrook et al. 1989), with uPA (F: 5'-CGGGGGGCTCTGTCACCTAC-3', R: 5'-CGGCCCGAGCTCACAATTC C-3') and PAI-1 primers (F: 5'-GGGAAAGGAGCCGTG GACCAG-3', R: 5'-GGGGCAGCCTGGTCATGTTG-3') in the same reaction. Reaction conditions were: 94 °C 5 min; 94 °C 30 s; 58 °C 30 s; 72 °C 1 min; 35 cycles; 72 °C 7 min (Miceli et al. 2005). Control PCR was done using HPRT primers (F: 5'-CTGCTGACA

AAGATTCACTGG-3', R: 5'-CCAAAGATGGTCAA GGTCGC-3') under conditions: 94 °C 5 min; 94 °C 30 s; 52 °C 30 s; 72 °C 1 min; 35 cycles; 72 °C 7 min. Conditions for uPAR PCR were: 94 °C 5 min; 94 °C 30 s; 60 °C 30 s; 72 °C 1 min; 36 cycles; 72 °C 7 min; with uPAR primers (F: 5'-CTGGAGCTGGTGGAGAAAAG-3', R: 5'-TGTTGCAGCATTTTCAGGAA G-3'). Determination of optimal number of cycles for each set of primers preceded the experiment, in order to allow the sequence amplification below saturation level. uPA and PAI-1 expression ratio was determined by densitometric analysis of bands obtained after multiplex PCR (ImageJ, NIH, Bethesda, MD, USA).

Comet assay

Cells analyzed by comet assay were treated with drugs for 24 h. Exposure to 1 mM H₂O₂ for 20 min served as a positive control. Comet assay was performed according to Singh et al. (1988) with slight modifications (15 min denaturation, 15 min electrophoresis at constant V = 24 V). Two slides were evaluated per sample. For each slide, 50 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 520/09 nm and a barrier filter of 610 nm (Axioplan Opton, Zeiss). A computerized image-analysis system (Comet version 5, Kinetic Imaging Ltd., Liverpool, UK) was used to measure percentage of tail DNA.

SDS PAGE and Western blot

Total cell extracts were prepared using lysis buffer (20 mM Tris-HCl pH = 7.5, 137 mM NaCl, 2 mM EDTA pH = 8.0, 10 % glycerol, 1 % Triton X-100, 100 μ g/mL phenylmethylsulfonyl fluoride (PMSF), 1 μ g/mL leupeptin, 1 μ g/mL aprotinin and 100 μ g/mL sodium vanadate) (Sambrook et al. 1989). Nuclear extracts were isolated according to Lee et al. (1994). Protein concentration was measured according to Bradford (Sambrook et al. 1989). 40 μ g of proteins were loaded on a 10 % SDS-PAGE gel and electrophoresed (Sambrook et al. 1989).

Proteins were transferred to PVDF membrane, blocked in 5 % nonfat dried milk and membrane probed with primary and secondary antibodies according to standard procedure (Sambrook et al. 1989). Bands were detected by chemoluminescence following manufacturer's instructions (GE Healthcare, Little

Chalfont, Buckinghamshire, UK). Equal loading of protein samples was validated by staining the membranes with Amido Black stain (Sambrook et al. 1989) and β -actin immunoblotting (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Primary antibodies recognized epitopes of phospho-JNK, JNK (both Becton-Dickinson, San Jose, CA, USA), c-Jun (Santa Cruz Technology), β -catenin (Sigma), YY1, phospho-GSK-3 α/β and GSK-3 β (all Cell Signaling Biotechnology, Danvers, MA, USA). Secondary antibodies were anti-rabbit IgG (Cell Signaling Technology) and anti-mouse IgG (Sigma), both HRP-linked. Densitometric analysis was performed using ImageJ (NIH, USA).

Statistical analysis

Results are presented as the mean value of certain number of replicas and are representative of at least two independent experiments. The deviation from mean value was expressed in the form of standard deviation (SD). Statistical analysis of data was performed using the software package STATISTICA 12.0 (StatSoft, Inc., Tulsa, OK, USA). The comparison between corresponding controls and treated samples (between themselves and individually) was done with one-way analysis of variance (one-way ANOVA) and post hoc Duncan's multiple range test. Differences were considered statistically significant at $p \leq 0.05$.

Results

Influence of PARP-1 inhibition on uPA induction after alkylation damage

A1235 glioblastoma cells are O⁶-methylguanine-DNA methyltransferase (MGMT) deficient and thus sensitive to alkylation damage caused by MNNG. We have previously shown that A1235 and other MGMT[−] cells specifically induce uPA protease activity under DNA alkylating conditions (Brdar 1986; Brdar and Matulic 1988). As poly(ADP-ribosyl)ation presents a parallel line of cell defense to alkylation induced DNA damage, our aim was to test if absence of PARP-1 activity will affect uPA proteolysis induction.

A1235 cells were treated with different concentrations of MNNG alone or in combination with PARP-1 inhibitor, 3-ABA. By preliminary analysis we

established that maximum uPA activity appeared when cells were allowed to grow for 24 h after treatment, and uPA activity measured in conditioned serum-free medium collected after additional 6 h incubation. The results are presented in Fig. 1a. The peak of uPA activity was observed in cells treated with 5 μ M MNNG. The addition of PARP-1 inhibitor increased uPA activity when added to 2 μ M MNNG, but decreased when MNNG concentration was 5 μ M or higher. Similar results were obtained using 20 μ M PJ-34, another PARP-1 inhibitor (data not shown).

In parallel to uPA activity measurements, DNA damage in treated cells was evaluated by the comet assay. The assay conditions allowed estimation of both, single and double strand DNA breaks, as a ratio of DNA in the tail. Figure 1b shows an increase in DNA damage by raising the MNNG concentrations. When applied simultaneously, PARP-1 inhibitor increased the level of DNA damage caused by MNNG. Only at drug concentrations above 20 μ M MNNG 3-ABA did not increase the level of DNA damage in comparison with MNNG treatment alone, but both treatments caused high level of DNA content in the tail reaching 80 %.

To see the effect of alkylation damage on the cells we measured, in parallel, cell viability, by means of the tetrazolium dye assay, 24 h after treatment. There was minimal (<10 %) loss of viability when treated with either MNNG, 3-ABA or both. Only at 20 μ M MNNG the number of viable cells decreased to 80 % (Fig. 2a). We obtained similar results by measuring the percentage of Trypan blue stained cells, as another viability test (Fig. 2b). Cell proliferation was estimated by counting the cells during 3 days after treatment. The growth curve revealed cell growth arrest after treatment with MNNG or in combination with PARP-1 inhibitor (Fig. 2c). In order to further investigate the treatment effect on our cells, we also performed SA- β -Gal test. The results showed increase in senescent cells during 5 days of treatment, although near the end of experiment we also observed the decrease in the cell number (Fig. 2d).

Influence of PARP-1 inhibition on uPA activity indicated that DNA damage could be the main mechanism of uPA induction. To explore this hypothesis, A1235 cells were transfected with plasmid carrying human PARP-1 cDNA, and clones with stable PARP-1 overexpression were examined. uPA activity was analyzed in PARP-1 transfected cells treated with increasing MNNG concentrations, in

Fig. 1 **a** uPA activity induction in A1235 cells after treatment with alkylating agent and PARP-1 inhibitor. Cells were treated with increasing concentrations of MNNG alone or in combination with 3-ABA. Conditioned serum-free medium was collected and uPA activity determined in quadruplicate. uPA activity was estimated by interpolation from a calibration curve of human uPA, normalized according to protein concentration of corresponding lysates and expressed as a fold multiplication of control cell values + SD. **b** DNA damage assessed by comet assay exposure to alkylating agent, PARP-1 inhibitor, and their combinations. 24 h after treatment cells were collected, lysed and electrophoresed under alkaline conditions. Differences in % tail DNA are presented as mean value based on three

replicates + SD. **c** Alkylation-induced uPA activity in A1235 and A1235 PARP-1 cells. A1235 PARP-1 cells were transfected with plasmid carrying PARP-1 cDNA. Cells were treated with different concentrations of MNNG for 24 h and their uPA activity was assayed as described before (**a**). In the columns marked with *asterisk* and *cross symbol* the mean values are significantly different from the control and their corresponding concentration of MNNG, respectively, according to Duncan test ($p \leq 0.05$). C, control; A, 3 mM 3-ABA; M2: 2 μM MNNG; M2 + A: 3-ABA + 2 μM MNNG; M5: 5 μM MNNG; M5 + A: 3-ABA + 5 μM MNNG; M10: 10 μM MNNG; M10 + A: 3-ABA + 10 μM MNNG; M20: 20 μM MNNG; M20 + A: 3-ABA + 20 μM MNNG

parallel with control A1235 cells (Fig. 1c). Caseinolysis showed that induction of uPA activity decreased in PARP-1 transfected cells, in comparison with control plasmid transfected and non-transfected cells, under the same alkylation conditions.

uPA system analysis

As the activity of the uPA system depends on the expression of uPA and its inhibitor PAI-1, we analyzed the level of mRNA expression, in control cells and cells treated 24 h with MNNG and PARP-

1 inhibitor. PAI-2 did not seem to be involved in uPA regulation of A1235 cells in these experimental conditions (data not shown). Total RNA was extracted from cells treated with either 5 μM MNNG, PARP-1 inhibitor, or both, and RT PCR performed with simultaneously added primers for uPA and PAI-1. Gel densitometric analysis revealed changes in uPA and PAI-1 ratio in treated samples, in comparison with untreated control, in favor of the increase in uPA activity (Fig. 3a). This effect was observed in the cells treated with either MNNG alone or combined with 3-ABA. Cells treated with

Fig. 2 **a** Cell viability of A1235 cells following the treatment with alkylating agent, PARP-1 inhibitor, and their combinations. Cells were treated with increasing concentrations of MNNG and 3 mM 3-ABA and after 24 h their viability was determined by XTT assay. The results are shown as mean values of three replicates + SD. **b** Trypan blue exclusion test. Cells were treated with 10 μ M MNNG, 3 mM 3-ABA, and their combination for different periods of time (1–5 days). At indicated time points we collected all cells, incubated them with 0.4 % Trypan blue solution and counted the cells on hemocytometer. The percentage of nonviable cells that took up the dye was calculated by counting at least 200 cells per treatment. The results are expressed as mean values of two replicates + SD. **c** Effect of alkylating damage and PARP-1 inhibition on cell proliferation. Cells were treated as described before (**b**) for different periods of time (1–3 days). Cell number was determined by counting the

cells electronically at daily intervals. Each point represents the mean value of three replicates $\pm$ SD. In time points marked by geometrical shapes mean that values are significantly different from the corresponding control according to Duncan test ($p \leq 0.05$). **d** SA- β -Gal staining of senescent cells. Cells were treated as described before (**b**) for 1, 2, 3 and 5 days, fixed in 1 % glutaraldehyde and assayed for SA- β -Gal. Senescent cells were counted on microscope and their number was expressed as a percentage of total cell population. 100–300 cells were counted per each treatment. The results are expressed as mean values of two replicates + SD. C, control; A, 3 mM 3-ABA; M2: 2 μ M MNNG; M2 + A: 3-ABA + 2 μ M MNNG; M5: 5 μ M MNNG; M5 + A: 3-ABA + 5 μ M MNNG; M10: 10 μ M MNNG; M10 + A: 3-ABA + 10 μ M MNNG; M20: 20 μ M MNNG; M20 + A: 3-ABA + 20 μ M MNNG

3-ABA alone showed increased mRNA levels of both, uPA and PAI-1, but their ratio remained the same as in control samples. uPAR expression was only slightly changed under the same experimental conditions (Fig. 3b).

Analysis of signaling pathways influenced by alkylation

Different signaling pathways are involved in uPA and PAI-1 expression regulation, and the whole system is

cell specific (Lengyel et al. 1995; Irigoyen et al. 1999; Parra et al. 2000; Nagamine et al. 2005). To investigate which pathways are involved in our system, we treated the cells with various inhibitors in combination with alkylation agent.

We employed a JNK inhibitor SP600125, an inositol phosphate kinases' inhibitor Wortmannin, a glycogen synthase kinase-3 (GSK-3) inhibitor LiCl and a cyclin-dependent kinase inhibitor Roscovitine. Cells were treated with drugs for 24 h and caseinolysis was performed with conditioned media collected after additional 6 h.

Fig. 3 uPA and PAI-1 expression ratio in A1235 cells treated with PARP-1 inhibitor and alkylating agent. 24 h after cell treatment total RNA was isolated and reversely transcribed. PCR was done with primers for uPA, PAI-1, uPAR and HPRT as control gene. **a** Relative level of expression of uPA and PAI-1, as obtained in multiplex PCR and determined by densitometry in

comparison with HPRT expression. Each bar represents the mean value + SD of at least two independent experiments. **b** uPA, PAI-1, uPAR and HPRT PCR products. C: control; A: 3 mM 3-ABA; M: 10 μM MNNG; M + A: 3 mM 3-ABA + 10 μM MNNG

Fig. 4 Inhibition of MNNG-induced uPA activity by different signaling inhibitors. Cells were treated with 5 μM MNNG alone or in combination with signaling inhibitors. After 24 h of treatment we collected conditioned serum-free media and determined uPA activity by radial caseinolysis as described before. Each bar represents the mean value of four replicates + SD. In the columns marked with *asterisk* the mean values are significantly different from the MNNG-induced uPA activity according to Duncan test ($p \leq 0.05$). M: uPA activity induced by 5 μM MNNG; M + SP: uPA activity induced by 5 μM MNNG in the presence of 10 μM SP600125; M + W: 5 μM MNNG and 1 μM Wortmannin; M + LiCl: 5 μM MNNG and 20 mM LiCl; M + R: 5 μM MNNG and 15 μM Roscovitine

In control cells treated only with different types of inhibitors, the basal level of uPA activity was the same as in control samples (Online Resource 1). Only treatment with GSK-3 inhibitor LiCl decreased uPA basal activity in untreated cells for more than 90 % (Online Resource 1).

Results presented in Fig. 4 show the effect of different signaling inhibitors on uPA activity, when applied concomitantly with MNNG in comparison with MNNG treatment alone. JNK and cyclin-dependent kinase inhibitors decreased uPA proteolysis for 75 %, GSK-3 kinase inhibitor for 90 % and inositol phosphate kinase inhibitor for 30 % (Fig. 4). These data indicate that the activity of different signaling pathways influence uPA system in A1235 cells after alkylation damage.

As JNK and c-Jun are common mediators of uPA induction (Parra et al. 2000), we analyzed their expression on the protein level, in the cells treated with MNNG and PARP-1 inhibitor for 1 and 24 h. Densitometric analysis showed a mild increase of phosphorylated JNK in samples treated with MNNG after 24 h. The signal was even weaker in samples with combined treatment (Fig. 5a). Samples treated with lower MNNG concentrations did not show significant JNK activation (data not shown). Similarly, we observed a mild increase in c-Jun expression in the nucleus, 24 h after treatment (Fig. 5c). Samples treated only with PARP-1 inhibitor for 24 h also weakly activated JNK and accumulated c-Jun in the nucleus (Fig. 5a, c).

Another pathway involved in uPA expression is PI3 kinase (PI3 K) pathway, converting to Wnt signaling on the point of GSK-3 complex (Seelan et al. 2008). As we observed inhibition of basal uPA activity after

Fig. 5 Effect of MNNG treatment and PARP-1 inhibition on signaling pathways. **a** Western blot analysis of phospho-JNK expression in cell extracts. Cells were treated with MNNG and/or 3-ABA for 1 and 24 h, respectively, and prepared whole-cell extracts subjected to immunoblot analysis. UVC radiated cells were used as positive control for JNK activation. **b** Phospho-GSK-3 α/β expression in cells treated with MNNG, 3-ABA and their combination. Cells were treated as described before (**a**) and their whole-cell extracts were analyzed by Western blot. β -actin expression was used as a loading control (**a**, **b**). **c** Analysis of

c-Jun and β -catenin expression in nucleus. Cells were treated as before (**a**) and after 24 h their nuclear extracts were analyzed. In order to validate equal loading of samples we stained the membrane with Amido Black and analyzed the expression of YY1. LiCl was used as positive control (**b**, **c**). All data were representative blots from at least two independent experiments. C: control; A: 3 mM 3-ABA; M: 10 μ M MNNG; M + A: 3 mM 3-ABA + 10 μ M MNNG; UVC: 0.0009 Js/cm² UV 245 nm; LiCl: 20 mM LiCl

treatment with LiCl, GSK-3 inhibitor, we explored GSK-3 α/β activity under experimental conditions. No significant differences were observed in GSK-3 activity between control and 24 h-treated cells, although LiCl treatment alone led to accumulation of phosphorylated GSK-3 (Fig. 5b). As downstream effector, nuclear β -catenin was also investigated and no significant changes in its expression were found in treated samples (Fig. 5c). As loading control we used YY1, polycomb protein reported to have stable expression in the nucleus (Rohwer et al. 2009).

Discussion

This work describes the modulation of uPA activity in glioblastoma cells under the influence of alkylation damage and inhibition of PARP-1 polymerase. A1235 cells, used in these experiments, do not have MGMT activity, and were shown to increase uPA activity after alkylation damage, like other mer- cells (defined as cells deficient in O⁶-methylguanine lesion repair)

(Scudiero et al. 1984; Brdar 1986). In the same experimental model it was demonstrated that the increase in MGMT repair capacity decreased uPA induction upon alkylation (Loncarek and Soric 1998). It was found that several other types of DNA damage also increased uPA activity: exposure of A1235 cells to double-strand break inducer etoposide, as well as UV treatment of Xeroderma pigmentosum fibroblasts (Miskin and Ben-Ishai 1981; Matulic and Brdar 2002). It is known that the cell has redundant repair mechanisms and unrepaired O⁶-methylguanine lesion could initiate mismatch repair (Knizhnik et al. 2013).

Here we explored the possible influence of another, parallel system involved in alkylation damage repair, the PARP-1 activation. PARP-1 activation is a consequence of single and double stranded DNA breaks and it recruits DNA repair molecules to the damaged site (Krishnakumar and Kraus 2010). Many studies showed PARP-1 activation after administration of alkylation agents, and PARP-1 inhibitors are employed in parallel with chemotherapeutics in BRCA (BRCAst CANCER)-deficient cells to increase

cell lethality (Farmer et al. 2005). Our results confirmed the involvement of PARP-1 in DNA damage repair, as its inhibition concomitant with MNNG treatment increased the level of DNA damage and decreased cell proliferation. Although high level of DNA damage was observed by comet assay, it could be ascribed to single strand DNA breaks: cell viability remained relatively high, though followed by cell cycle arrest. Knizhnik et al. (2013) also found that DNA damage repair deficient cells, when treated with certain drugs, postpone apoptosis to second cell cycle.

Mechanisms of uPA system induction upon DNA damage in glioblastoma cells are still not fully explained. On the molecular level, uPA and PAI-1 transcription is regulated through a number of signaling pathways, and some of them are activated as a consequence of cellular stress (Parra et al. 2000; Nagamine et al. 2005; Vidal et al. 2005; Shetty et al. 2008b). In addition to that, we show here that induction of uPA activity can be modulated by PARP-1 inhibition. There are several possibilities in which PARP-1 could influence uPA system. PARP-1 can modify transcription factor activity in pathways regulating uPA and PAI-1, such as Sp1, NF κ B, p53, Ets-1 and AP1 (von der Ahe et al. 1988; Lee et al. 1994; Lengyel et al. 1996; Wang et al. 1998; Ibanez-Tallon et al. 1999; Nagamine et al. 2005; Shetty et al. 2008a; Huang et al. 2009; Krishnakumar and Kraus 2010; Legrand et al. 2013; Yang et al. 2013). Regulation of uPA and PARP system was shown to be interdependent in experiments done by Caldini and his group. uPA expression induced by FGF2 was increased by PARP activated through MAP kinase pathway and in other system PARP activity inhibition decreased uPA activity (Caldini et al. 2005, 2011). In A1235 cells PARP-1 inhibitor alone did not significantly change the growth rate and viability under our experimental conditions. We observed small increase in both, uPA and PAI-1 expression, but there were no changes in uPA activity compared with control. There was also increase in c-Jun expression and weak activity of JNK after 24 h treatment with PARP-1 inhibitor. PARP-1 has many roles in the regulation of transcription acting through protein–protein interactions and modifications of transcription factors and as some of these activities require activated PARP-1 it is possible that they could be influenced by PARP-1 inhibition (Krishnakumar and Kraus 2010). PARP-1 can also be activated through several signaling pathways (Huang et al.

2009, Caldini et al. 2011) and could even effect the level of DNA damage through activity of Ets-1, as shown in Legrand et al. (2013). However, we concluded from Legrand's and our experiments, that influence of activated PARP-1 on DNA damage repair was dominant over transcription factor modification.

Our results indicate that there is also another possible pathway of PARP-1 involvement; indirect influence on the level of DNA damage. According to our experimental model, in which PARP-1 inhibition did both, increase and decrease of uPA proteolysis, we conclude that PARP-1 influences the system by its DNA damage repair activities. Besides that, our previous results (Brdar and Matulic 1988, unpublished results), such as specific cell-type dependent conditions of optimal uPA induction, delayed reaction (maximum uPA activity 24 h after DNA damage), narrow range of MNNG concentrations able to induce uPA connected with relatively high viability and growth arrest, and MGMT[−] intracellular milieu, led us to hypothesize that cells attempt to reprogram and/or differentiate and that these processes involve uPA induction. According to this hypothesis, PARP-1 inhibition “titrated” this narrow range of DNA damage optimal for these processes. Decrease in uPA activity observed under conditions of higher level of DNA damage could be a consequence of increased cell stress and activation of some other cellular programs. When higher PARP-1 expression increased the capacity of alkylation damage repair, uPA activity induction decreased and the maximum induction was shifted toward higher MNNG concentrations.

Furthermore, our experiments showed that the increase in uPA activity upon alkylation was a consequence of the uPA/PAI-1 ratio change. This is in agreement with Hagelgans et al. (2013) findings showing the importance of uPA/PAI-1 balance in regulating cell migration and invasive growth capabilities. Recently Sanchez-Tillo et al. (2013) found that ZEB-1, one of the master factors in epithelial-to-mesenchymal transition, has the ability to coordinate uPA and PAI-1 expression: at the level of transcription and mRNA stability, respectively. The aim of these processes is to increase cell protease activity during conversion to mesenchymal phenotype (Sanchez-Tillo et al. 2013). We suppose that specific DNA damaging conditions in our glioblastoma cells could initiate a similar program. It is known that some carcinogens induce differentiation at low concentrations in specific

cell lines (Shetty et al. 2008a, b; Zhang et al. 2008; Sherman et al. 2011; Santos et al. 2014). In addition to that, our preliminary experiments show that ZEB-1 overexpression in A1235 cells increased the basal uPA activity and abrogated its induction upon alkylation (unpublished results).

Reprogramming trial could also explain activation of a plethora of signaling pathways whose inhibition decreased uPA activity induction. Several signaling pathways, like MAP kinases, phosphoinositide 3-kinase (PI3 K) survival pathways, Wnt, p53, etc. have different regulation patterns depending on the cell type (Whitley et al. 2007; He et al. 2010; Moreau et al. 2011). In our experiments we show that inhibition of several signaling pathways modulates uPA activity. The strongest is the effect of LiCl, a GSK-3 inhibitor, which nearly abrogated uPA activity. We observed constitutively active GSK-3 α and weak activation of GSK-3 β , as well as control levels of β -catenin in the nuclei of treated cells. Furthermore, as PI3 K is often constitutively active in glioblastomas, inhibition of this signaling could explain uPA down-regulation (Zhang et al. 2008). Parra et al. (2000) found uPA induction in human cells after MNNG treatment to be dependent on the activation of JNK pathway. We showed here that JNK inhibitor decreased uPA activity, but did not abrogate it. JNK activation in our experimental conditions appeared to be mild and Jun expression was also only slightly changed in MNNG-treated cells.

It was also shown that uPA induction could be dependent on the cell cycle and cyclin dependent kinases (Zavizion et al. 1998). We found that agents, like hydroxyurea and serum depleted medium (unpublished results), causing cell cycle arrest alone, did not exert significant influence on uPA induction, as well as a cdk inhibitor, Roscovitine alone. Only specific conditions of DNA damage enabled Roscovitine to inhibit uPA activity.

Interestingly, Wortmannin, an inhibitor of DNA break sensor ATM kinase, does not exert strong influence on the uPA activity. It is possible that some other ATM-related kinases, insensitive to this inhibitor, are involved, as described by others (Vidal et al. 2005; Noonan et al. 2012).

Although inhibitors of different signaling pathways strongly inhibited uPA activity, we did not detect strong activation of either of signaling proteins explored. We propose that low level of DNA damage

and consequences of repair trial weakly activate many signaling pathways which act synergistically and in specific combination, so inhibition of each of them can influence complex intracellular reprogramming.

In conclusion, our results suggest that PARP-1 inhibition modulates uPA activity in glioblastoma cells after alkylation damage by inhibition of DNA repair. Moreover, they indicate that increase in uPA activity might be a consequence of the change in the balance between uPA and PAI-1 level. Increase in uPA activity could be a consequence of complex intracellular reprogramming specific for glioblastoma cells, triggered by DNA damage. Further experiments are needed to elucidate the mechanism of this reprogramming in our model cells.

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Conflict of interest The authors declare that there is no conflict of interest.

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