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Urokinase-type Plasminogen Activator and Plasminogen Activator Inhibitor Induction by Etoposide in a Glioblastoma Cell Strain

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Summary

Urokinase plasminogen activator (uPA) and its inhibitors (PAI) are elements of plasminogen activation system, a proteolytic system involved in many physiological and patological processes. In this paper their induction by etoposide, a topoisomerase II inhibitor, in A1235 glioblastoma cell strain is described. Etoposide induced uPA and PAI production, in a dose- and time-dependent manner. Induction was based on the activation of their promoters and extracellular proteolysis was dependent on their equilibrium. Etoposide caused p53 activation and p21 and *gadd45* induction, which could be responsible for the cell growth arrest. Our data indicate that several pathways could be involved in the uPA and PAI induction.

Key words: urokinase, plasminogen activator inhibitor, uPA promoter, PAI promoter, etoposide, glioblastoma cell strain

Introduction

Urokinase-type plasminogen activator (uPA) is highly specific serine protease involved in the regulation of many normal and malignant processes, participating in tissue remodelling, migration and invasion *(1)*. It is also involved in non-proteolytic signalling events leading to adhesion and chemotaxis *(2)*. uPA converts plasminogen to plasmin, which further degrades extracellular matrix and activates latent collagenases. The ubiquitous and with wide-spectrum of substrates, plasmin could be dangerous as a protease if not tightly controlled through plasminogen activator activation. Plasminogen activation system has a multistep control through uPA gene transcription, protein production, localized activation, as well as its inhibition and degradation *(3)*. uPA is secreted as a single-chain proenzyme and converted by limited proteolysis into an active two-chain molecule. Active uPA can bind to its cell surface receptor (PAR), and to its inhibitors, PAI-1 and PAI-2. uPA-PAI complex bound to receptor is internalized and degraded *(4)*.

Plasminogen activation system is closely associated with tumour invasiveness and metastasis by virtue of its biological properties, and uPA and PAI were found to be overregulated in tumour or neighbouring cells *(3)*. Both of them can be induced with a number of growth factors, hormons, oncoproteins, cytokines, but also with some tumour promoters and carcinogens *(5–9)*, all of them activating specific signal transduction pathways. In certain cell lines uPA induction was shown to be connected with unrepaired DNA damage: methyl-nitroso urea and N-methyl-N'-nitro-N-nitroso-guanidine caused uPA induction in alkylation-repair deficient cell strains *(10)* and low UV influences in xeroderma pigmentosum DNA repair-deficient human cells *(11)*. In this context it was interesting to analyse plasminogen activation system in A1235 glioblastoma cell strain after treatment

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with topoisomerase II inhibitor etoposide *(12)*. This widely used anticancer drug poisons the topoisomerase II, induces double strand breaks and causes mutagenic and lethal events. Here we show that it can also induce uPA and PAI production in a dose-dependent manner. Used as chemotherapeutics, etoposide could induce similar processes *in vivo*.

Material and Methods

Plasmids, cells and chemicals

Plasmid 5.5kb-uPACAT, containing human 5.5 kb uPA promoter fused to the CAT gene and PAI-1CAT, containing 633 bp long human PAI-1 promoter, were obtained from Dr Y. Nagamine (Friedrich Miescher Institute, Basel, Switzerland). Plasmid pHUL45B2, containing *gadd45* gene was a kind gift from Dr A. Fornace (NCI, NIH, Bethesda, MD). Plasmid phPAI-1 05, used for a hybridization probe, was a kind gift from Dr P. Autzen Usher and Dr K. Dano (Finsen Laboratory, Copenhagen, Denmark), and pNATCAT, containing human PAI-2 promoter from Dr T. M. Antalis (Queensland Institute of Medical Research, Brisbane, Australia). Plasmids were purified by alkaline lysis method as described previously *(13)*.

Human glioblastoma cell line A1235, obtained from S. A. Aaronson (NCI, NIH, Bethesda, MD), and its transfectants were cultivated in DMEM (Sigma Chemicals, St. Louis, MO) supplemented with 10 % fetal bovine serum (Gibco Life Technologies, UK) and antibiotics, at 37 °C and 5 % CO_2. Cell counts were performed by Coulter Counter.

Antibodies

p53 antibodies, provided by Dr L. Banks (ICGEB, Trieste, Italy), were obtained from conditioned media of three clones producing monoclonal antibodies (pAB1801, pAB1802, pAB1803) *(14)*. Monoclonal mouse antibody p21 (WAF 1/Ab-1) was supplied from Oncogene Research Product Calbiochem, La Jolla, CA and human uPA monoclonal mouse antibody from American Diagnostica, US.

Chemicals and enzymes

Human urokinase was purchased from Calbiochem and human plasminogen from KabiVitrum (Stockholm, Sweden). D-threo-[dichloroacetyl-1-^{14}C]Chloramphenicol was purchased from Amersham (UK) and acetyl coenzime A from Boehringer Meinheim (Germany). Adriamycin was supplied from Farmitalia Carlo Erba, Italia. Etoposide (Etopol, Krka, Novo Mesto, Slovenia) was dissolved in physiological solution.

Transfection and CAT assay

A1235 cells transfected with 5.5 kb-uPACAT plasmid, were described previously *(15)*. PAI-1CAT and PAI-2CAT clones were obtained by stable transfection with PAI-1CAT and pNATCAT plasmids, respectively, using Superfect Transfection Reagent (Qiagen, Germany). During the experiment, cells were treated with etoposide for 24 h and incubated in the drug-free medium for additional 24 h when cell extracts were collected. Cell extracts were prepared and assayed for the chloramphenicol acetyltransferase (CAT) activity according to a standard procedure *(13)*. Briefly, the same amount of cellular extracts was incubated with 266 μM acetyl coenzyme A and 0.5 μCi [^{14}C]chloramphenicol for 1–1.30 h at 37 °C, and after the ethyl acetate extraction separated by thin-layered chromatography. After the exposure, bands on X ray film were analysed by Image Master VSD Software (Pharmacia Biotech, Uppsala, Sweden).

PAGE and Western blot

Total cell extracts were prepared using lysis buffer (50 mM Tris HCl pH=8.0, 150 mM NaCl, 0.02 % Na azide, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1 % Triton X-100) and protein concentration was determined according to Bradford *(13)*. Protein samples were mixed with the loading buffer, boiled at 95 °C for 5 min (for denaturing conditions), loaded on a 12 % SDS-PA gel and electrophoresed as described previously *(13)*. Proteins were transferred onto nitrocellulose membrane (Hybond, Amersham) in transfer buffer (25 mM Tris HCl pH=8.3, 192 mM glycin, 20 % methanol), employing Hoefer (Amersham-Pharmacia) transblot apparatus. Blots were blocked overnight in PBST (0.1 % Tween-20 in phosphate buffered saline) containing 5 % nonfat dried milk and incubated with primary antibodies to p53 or p21 for 1–2 h. As a secondary antibody biotinylated goat-antimouse IgG (Oncogene Research Products, Calbiochem) was used, followed by incubation with streptavidin, H_2O_2 and 3.3'-diaminobenzidine (DAB) *(13)*. For uPA analysis, conditioned media were concentrated using Microcon 10 tubes (Amicon, USA). Gels were run under nondenaturing conditions, membranes blocked in TBST (0.5 % Tween-20 in Tris buffered saline) containing 5 % nonfat dried milk and primary mouse uPA antibody was used (American Diagnostica, USA). For detection, ECL system (Amersham) was employed.

Plasminogen activator and plasminogen activator inhibitor activity analysis

uPA activity was assayed by radial caseinolysis in the conditioned, serum-depleted media from etoposide treated A1235. Medium aliquotes were added to the wells made on the 1 % agarose plate containing 2.5 % nonfat powdered milk, 0.1 % sodium azide and 5 μg/mL human plasminogen. Diameters of clear areas were measured after incubation at 37 °C for 24 h and uPA activity estimated by interpolation from a calibrated curve of human urokinase *(16)*.

PAI-1 activity was assayed by reverse casein zymography. Exponentially growing A1235 cells were treated with etoposide or adriamycin for 24 h, when medium was replaced for the serum- and drug-free one, for additional 10 h. The conditioned media were collected and concentrated by centrifugation in Centricon 30 tubes. Cell lysates were prepared as previously described *(17)*, and the amount of protein determined according to Bradford *(13)*. Volumes of conditioned media were normalized and run on 12 % polyacrylamide gel under nondenaturing conditions. After electrophoresis gels

were incubated in 2.5 % Triton X-100 for 2–3 h and rinsed in water for 1 h. For uPA detection gel was put on casein underlay (1 % agarose, 3–4 % non fat milk, sodium azide and 12.5 μg/mL human plasminogen) *(10)*. For PAI-1 detection parallel gel was put on casein underlay containing 0.05 U/mL human urokinase *(18)*. Gels were incubated for 24 h at 37 °C and photographed.

RNA extraction and Northern blot analysis

Total RNA was extracted from cultured cells as previously described *(19)*. RNA samples were separated by agarose gel electrophoresis, capillary transferred to a nylon membrane (Roche Molecular Biochemicals, Germany) overnight, and crosslinked with UV. For *gadd45* detection, the membrane was hybridized at 65 °C overnight with the probe labelled with [^{32}P] by random priming reaction and exposed to X-ray film at –70 °C. For PAI-1 detection, the membrane was hybridized at 65 °C overnight with the probe labelled with digoxigenin by random priming reaction, according to manufacturer's manual (DIG DNA labeling and detection kit, Roche Molecular Biochemicals). Detection was performed using CDP-Star chemiluminescence substrate (Roche Molecular Biochemicals). X ray films were analysed by Image Master VSD Software.

Results

Etoposide enhances uPA, PAI-1 and PAI-2 promoter activity

We examined the effects of etoposide on the production of urokinase and its inhibitors (PAI-1 and PAI-2) in A1235 cells by analysing their promoter activities. Cells permanently transfected with either 5.5 kb uPA promoter-CAT or PAI-1 promoter-CAT or PAI-2 promoter-CAT plasmids were treated with different concentrations of etoposide for 24 h, incubated in the drug-free medium for additional 24 h and analysed for CAT activity. As shown in Fig. 1, uPA, PAI-1 and PAI-2 promoters were all activated by etoposide treatment in a dose-dependent manner.

Etoposide induces uPA and PAI-1 production

To determine whether etoposide-induced uPA and PAI promoter activities are accompanied with extracellular urokinase production, A1235 cells were exposed to a 2.5–10 μM concentration range of etoposide for 6, 12 or 24 h in the serum-free medium and the excreted uPA analysed by Western blots and caseinolysis. As illustrated in Fig. 2A and 2B, etoposide induced several-fold control level of uPA production in a dose- and time-dependent manner, when assayed by Western blotting. The extracellular uPA of both etoposide-treated and untreated cells comigrated with commercial 55 kDa and, in some samples, with 30 kDa human urokinase; a band of approximately 90–100 kDa was also observed, apparently induced in a dose- and time-dependent manner.

Radial caseinolysis performed with the same samples revealed that a 24-hour exposure of A1235 cells to 5 μM etoposide enhanced 5-fold control level of uPA activity, whereas 10 μM etoposide enhanced uPA activity only 2-fold (Fig. 2C). These findings are of interest for two reasons: (*i*) drop in uPA activity that was observed at 10 μM etoposide coincided with the accumulation of the slow moving 90–100 kDa band (Fig. 2A and 2B) (*ii*) this band could represent a complex between uPA and its inhibitors, PAI-1 or PAI-2 *(20)*. Since net uPA activity is also determined by PAI-activity *(21,22)*, we studied the effect of etoposide on PAI-1 production in A1235 cells. The cells were treated with 10 μM etoposide for 2, 6, 12 and 24 h, when total RNA was extracted and subjected to Northern blot analysis, using digoxigenin labelled PAI-1 probe. As shown in Fig. 3, etoposide induced several-fold control level of two PAI-1 RNA species (2.4 kb and 3.4 kb) in a time-dependent manner *(23)*.

To further evaluate etoposide mediated induction of PAI-1 mRNA, the effect on the extracellular PAI-1 production was also examined. The cells were treated with either 5 μM etoposide or 2.5 μM adriamycin (which is also a topo II inhibitor and was used here as a positive

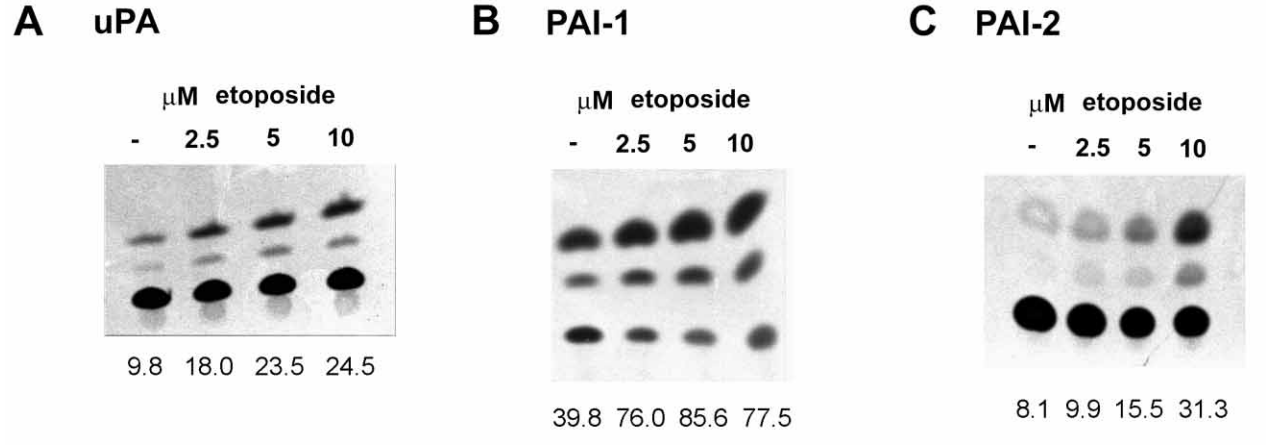

Fig. 1. Etoposide enhances uPA, PAI-1 and PAI-2 promoter activity. A1235 cells were permanently transfected with human 5.5 kb uPA promoter-CAT reporter plasmid (A), PAI-1 (B) and PAI-2 promoter CAT reporter plasmids (C). Cells were treated with different etoposide concentrations for 24 h, washed and incubated for additional 24 h in the drug-free medium, when cell extracts were collected and CAT assay performed. Numbers below figures represent percentage of chloramphenicol conversion (CAT activity).

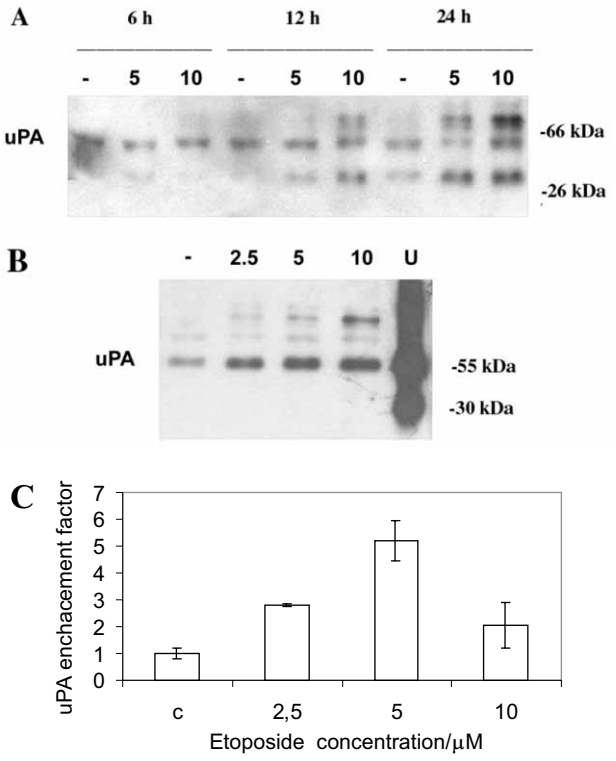

Fig. 2. Etoposide induces extracellular uPA production. A1235 cells were treated with 5 and 10 μM etoposide for 6, 12 and 24 h (A) or with 2.5, 5 and 10 μM etoposide for 24 h (B), in the serum depleted medium. At indicated time points conditioned media were collected, concentrated and subjected to Western blot analysis (A and B) or radial caseinolysis (C). As internal molecular weight standard, commercial human urokinase was used (U).

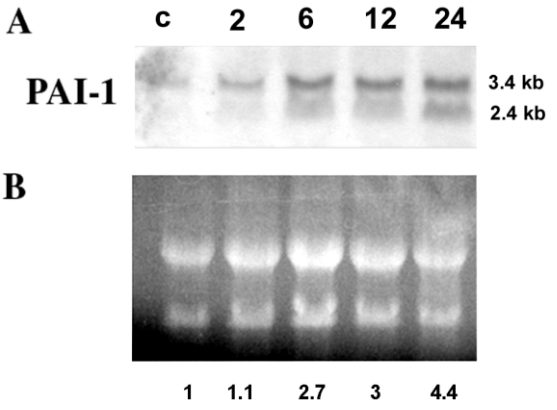

Fig. 3. Etoposide induces PAI-1 mRNA. A1235 cells were treated with etoposide for 2, 6, 12 and 24 h, when total RNA was isolated, transferred on the nylon membrane and hybridized with digoxigenin labelled PAI-1 probe. Detection was performed by chemiluminiscence reaction. **A**. Northern blot: **c**, control; **2**, 2 h etoposide treatment; **6**, 6 h treatment; **12**, 12 h treatment; **24**, 24 h treatment. **B**. Gel stained with ethidium bromide. Numbers below figure represent relative intensity of the bands.

control) for 24 h and incubated in the serum depleted medium for additional 10 h. Conditioned media were

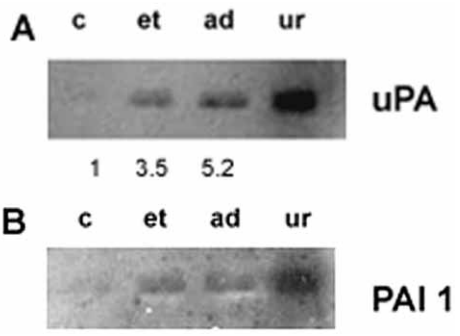

Fig. 4. Etoposide induces uPA and PAI-1 activity. Cells were treated with 5 μM etoposide or 2.5 μM adriamycin for 24 h, and incubated in the medium without serum for additional 10 h when conditioned medium was collected, concentrated, normalized, and analysed by PAGE. The gel was incubated at 37 °C for 24 h on the second indicator gel containing either human plasminogen (A), or human plasminogen and 0.05 U/mL urokinase (B) for PAI-1 detection. **c**, control; **ad**, 2.5 μM adriamycin; **et**, 5 μM etoposide; **ur**, 0.01 U/μL urokinase. Numbers below the gel represent relative intensity of the bands.

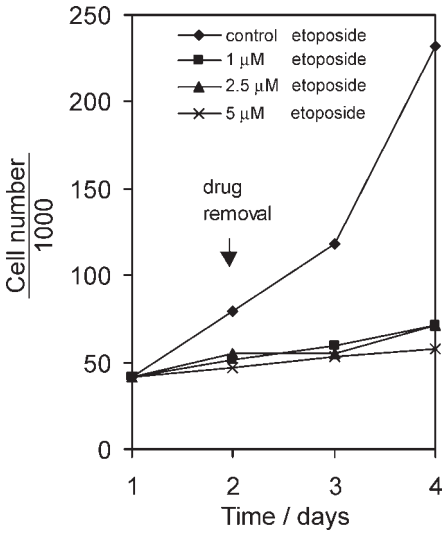

Fig. 5. Effect of etoposide on the growth of A1235 cells. Cells were treated with indicated concentrations of etoposide for 24 h and incubated in the drug-free medium until the end of the experiment. Cell number was determined electronically at daily intervals.

collected, electrophoresed and subjected to reverse gel zymography using either the casein-plasminogen underlay (for uPA detection) or the casein-plasminogen-urokinase underlay (for PAI-1 detection). Zymography revealed strong uPA lysis zones in the samples obtained from etoposide and adriamycin treated cells, as compared with untreated control (Fig. 4A), indicating that both topo II inhibitors induced high levels of uPA. Zymographic analysis of the same samples using casein-plasminogen-urokinase underlay showed unlysed zones just below urokinase bands (Fig. 4B), indicating the presence of increased amounts of PAI-1 in the samples obtained from the drug-treated cells. PAI-2 could not be detected by this method due to its instability *(24)*.

Etoposide stabilizes p53 and induces gadd45 and p21

In the view of the above observations it was desirable to obtain information concerning the effect of etoposide on the cell growth under the treatment conditions that caused uPA and PAI induction. uPA and PAI induction occurred at etoposide concentrations of 2.5–5 μM which caused cell growth arrest, but without obviuos cell dying (Fig. 5). However, etoposide at concentrations above 10 μM and after prolonged treatment (*e.g.* 50 h) caused apoptosis (data not shown).

Since etoposide is known to cause p53 stabilization and p53-dependent p21 and *gadd45* induction, we wanted to determine whether genes that are involved in cell growth arrest and apoptosis *(12,25)* could also be induced in A1235 cells. Extracts were prepared from cells treated for 4 h with 5 μM etoposide or 5 μM adriamycin (a positive control) *(12)* and subjected to Western blot analysis. As shown in Fig. 6A and 6B, etoposide and adriamycin induced high levels of both p53 and p21 as compared to the untreated controls. As *gadd45* is often induced by p53 and is also involved in the growth arrest *(26)*, RNA prepared from the cells treated with 5 μM etoposide or 5 μM adriamicin for 10 h was analysed by Northern blot. As shown in Fig. 6C, *gadd45* gene was strongly induced in the treated cells.

Discussion

Plasminogen activation system involves several genes which play an important role in biological processes and thus are regulated through the whole net of signal transduction pathways. We examined the ability of etoposide, topoisomerase II inhibitor, to induce the production of uPA, PAI-1 and PAI-2 in A1235 glioblastoma cell strain. The data show that etoposide induced uPA and PAI in a dose- and time-dependent manner, and that this induction was based on the activation of respective promoters. Western blot analysis showed that conditioned media of etoposide-treated cells, beside 30–55 kDa urokinase species, contain a slow moving 90–100 kDa form, which coincided with a drop of uPA activity (Fig. 2). Since etoposide also induced PAI production in A1235 cells, it was assumed that this band could represent a complex between uPA and its inhibitors *(23,27)*. This could be reconciled with the observation that net uPA activity is also determined by PAI activity *(21,22)*.

Etoposide is widely used as chemotherapeutic drug and many mechanisms of its action are known *(12)*. As a topoisomerase II poison, it makes DNA adducts and stops DNA polymerase, and as a consequence, double strand breaks can occur. In response to DNA damage, p53 is activated and ATM kinase recruited. p53 effects cell cycle arrest after activating *p21, gadd45* and *mdm2* genes to allow DNA repair to take place *(26,28)*. We showed that etoposide activated p53 and induced p21 and *gadd45* in A1235 cells that could cause observed cell growth arrest of treated cells. Only doses higher than 10 μM or prolonged treatment probably activate p53-dependent pathways connected with apoptosis, as in the case of unsuccessful repair *(28)*. In comparison with other topo II inhibitors, etoposide is specific for induction of significant dose-dependent DNA damage and double strand breaks at concentrations that do not affect cell survival *(29)*.

p53 could be connected with PAI-1 induction after etoposide treatment. PAI-1 promoter contains p53 binding site responsible for PAI transcription after p53 overexpression *(30)*. Etoposide induced PAI-1 expression co-

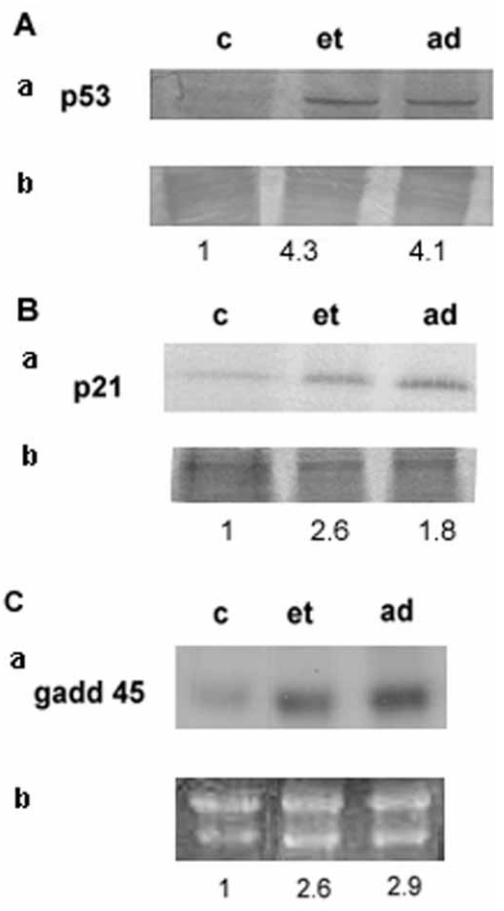

Fig. 6.A. Etoposide activates p53. A1235 cells were treated with 5 μM etoposide or 5 μM adriamycin for 4 h when they were collected, extracts prepared and analysed by Western blotting with p53 antibody.
c, control; **et**, 5 μM etoposide; **ad**, 5 μM adriamycin.
a. Membrane immunoblotted with p53 antibody and stained with biotin-horseradish peroxidase-DAB. **b**. Coomassie blue stained gel.
Fig. 6.B. Etoposide and adriamycin induce p21. Experimental conditions were as in Fig. 6A, except that p21 antibody was used.
c, control; **et**, 5 μM etoposide; **ad**, 5 μM adriamycin.
a. Membrane immunoblotted with p21 antibody and stained with biotin-horseradish peroxidase-DAB. **b**. Coomassie blue stained gel. Numbers above the figures represent induction factors relative to the control, as determined by densitometry.
Fig. 6.C. Etoposide and adriamycin induce expression of *gadd45* mRNA in A1235 cells. A1235 cells were treated with 5 μM etoposide or 5 μM adriamycin for 10 h, when total RNA was extracted, electrophoresed and subjected to Northern blot analysis. The membrane was hybridized with [^{32}P]*gadd45* and autoradiographed.
c, control; **et**, etoposide; **ad**, adriamycin.
a. Northern blot hybridized with *gadd45* probe.
b. Gel stained with etidium bromide.
Numbers below the gels represent relative intensity of the bands.

incided with p53 activation. Considering uPA induction, Kunz showed that overregulation of p53 can inhibit uPA transcription, although the mechanism is still unknown *(30)*. Therefore, the pathways leading to etoposide induced uPA production could be different. uPA induction is usually connected with several signaling pathways; the best known are those involving TPA and protein kinase C activation, phosphatase inhibition or cytoskeletal disruption *(5,9,31–33)*. The last step usually includes AP-1 activation and binding of transcription factors to one or both of AP-1 sites on the uPA promoter. Etoposide was found to induce JNK and c-jun phosphorylation and protein kinase C activation *(34–36)*. In the most cell lines its action was examined in the context of apoptosis: the role of c-jun was found to be cell type specific and different apoptotic pathways were found to operate in different cell lines *(35,37,38)*. We observed up to two-fold activation of the TRE promoter (containing 5xAP-1 binding sequence) by etoposide and adriamycin treatment (data not shown). On the other side, curcumin (AP-1 inhibitor) *(39)* did not inhibit etoposide-induced uPA promoter in A1235 cells (data not shown). AP-1 induction cannot always be corellated with uPA induction: some strong inducers and upstream oncoproteins did not significantly change uPA level (*e.g.* pyrrolidine dithiocarbamate) *(15)*, and adriamycin, a strong AP-1 inducer and also topoisomerase II inhibitor, did not show significantly stronger effect on the uPA promoter activation in comparison with etoposide (data not shown). In addition, AP-1 elements are present also in the PAI-1 and PAI-2 promoters *(20,27)*, and could contribute to their induction. Whether AP-1 activation is the mechanism of uPA promoter activation or there are some other elements included, remains to be elucidated.

uPA and PAI, as elements of plasminogen activation system, play a crucial role in the regulation of extracellular matrix degradation in cancer invasion and metastasis. They are often upregulated in cancer or neighbouring cells and could even be prognostic factors *(40)*. Concomitant expression of uPA and PAI was found to increase in different cancer cell lines *(40,41)*; oncogenic stimuli may activate constitutively their upstream pathways *(3)*. For instance, many tumour promoters and carcinogens are known as uPA and PAI inducers, TPA being one of the best studied *(1,8,11,42)*. Carcinoma cells treated chronically with increasing concentrations of adriamycin, showed overregulation of PAI-1 level in comparison with untreated (sensitive) cells *(43)*. A1235 cells strongly expressed uPA after the treatment with alkylating agents, as they are defective in the repair of that kind of damage *(10)*. These cells also, treated with cisplatin, produced increased levels of extracellular uPA and PAI-1 *(44)*.

In conclusion, our results suggest that etoposide induced uPA and PAI production in A1235 glioblastoma cells is based on the activation of their promoters and probably associated with DNA damage dependent p53 activation. Since etoposide is a widely used chemotherapeutic agent, these data indicate that similar processes could occur *in vivo*.

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Indukcija urokinaznog tipa plazminogenskog aktivatora i njegova inhibitora etopozidom u glioblastomskoj staničnoj liniji A1235

Sažetak

Urokinazni tip plazminogenskog aktivatora (uPA) i njegovi inhibitori (PAI) dijelovi su plazminogenskog aktivacijskog sustava, proteolitičkog sustava uključenog u mnoge fiziološke i patološke procese. U ovom je radu opisana njihova indukcija etopozidom, inhibitorom topoizomeraze II, u stanicama glioblastoma A1235. Etopozid je inducirao uPA i PAI ovisno o koncentraciji i vremenu inkubacije. Indukcija je ovisila o aktivaciji njihova promotora, a ekstracelularna proteoliza o njihovoj ravnoteži. Etopozid je uzrokovao aktivaciju p53, kao i indukciju p21 i *gadd45*, te bi oni mogli biti odgovorni za zaustavljanje staničnog rasta. Rezultati upućuju da bi nekoliko signalnih puteva moglo sudjelovati u indukciji uPA i PAI.