Effect of Ukrain on matrix metalloproteinase-2 and Secreted Protein Acidic and Rich in Cysteine (SPARC) expression in human glioblastoma cells

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Glioblastoma is a highly malignant brain tumor with a highly invasive phenotype and hence an unfavorable prognosis even in response to multidisciplinary treatment strategies. Ukrain, a semi-synthetic thiophosphoric acid derivative of the purified alkaloid chelidonine, has been used in the therapy of several solid tumors, but little is known about its effect on glioblastoma and, in general, about the molecular mechanisms responsible for its effects. We used RT-PCR, Western blot and SDS-zymography to investigate the effects of three doses of Ukrain (0.1, 1 and 10 μmol/l) on the expression of genes and proteins involved in the extracellular matrix remodeling associated with tumor invasion in human cultured glioblastoma cells treated for 24, 48 and 72 h. We analyzed the expression of matrix metalloproteinase-2 and -9, the main mediators of glioblastoma invasiveness, and secreted protein acidic and rich in cysteine (SPARC), involved in the regulation of cell–matrix interactions. There was a significant, dose-related decrease of glioblastoma cell proliferation and a tendency to downregulation of SPARC at the protein level 72 h after 10 μmol/l Ukrain, suggesting the drug may be a useful therapeutic tool for brain tumors. *Anti-Cancer Drugs* 17:189–194 © 2006 Lippincott Williams & Wilkins.

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Introduction

Ukrain (NSC 631570) is purified water-soluble alkaloids isolated from greater celandine (*Chelidonium majus* L.), a member of the Papaveracee family [1]. It is employed as an anti-cancer agent, with high cytotoxic activity on malignant cells [2,3]. Ukrain is also an immunostimulating and immunomodulating compound [4], and has anti-angiogenic effects on human endothelial cells *in vitro* [5].

Over the past years plant extracts such as Ukrain have been investigated as new anti-tumoral drugs in the search for therapeutic strategies for malignancies. Ukrain acts both as a single agent and in combination with conventional chemotherapy, and there are case reports of responses for a range of solid tumors including the colon, rectum, breast, pancreas, bladder and ovary [6–10]. To our knowledge, however, little information is available on the treatment of brain tumors such as gliomas.

Among brain tumors, one of the leading causes of death among young children and adults, gliomas are the most common primary brain malignancies, accounting for more than 40% of all central nervous system neoplasms [11], and glioblastoma is a highly malignant glioma typically affecting adults between 45 and 60 years of age [12]. With its highly invasive phenotype, glioblastoma infiltrates diffusely into regions of the normal brain, making total surgical removal impossible; therefore, patients have a poor prognosis, even in response to multidisciplinary treatment strategies including surgery, radiotherapy and chemotherapy [13,14].

The physical processes of tumor invasion, that involve disengagement of the cells from their microenvironment, followed by breakdown of the surrounding matrix, cell movement and re-establishment of the local environment at a new site, enable glioblastoma cells at the tumor-invasive front to overcome the extracellular matrix (ECM) barrier and penetrate adjacent brain structures. This is accomplished by an ECM remodeling process involving matrix metalloproteinases (MMPs) and secreted protein acidic and rich in cysteine (SPARC) [15–17].

MMPs are a family of zinc-dependent, tumor cell-secreted proteolytic enzymes that break down ECM components [18,19]. Their expression in gliomas correlates the tumor invasiveness with the proteolytic activity of MMP [20–22]. Gelatinases, particularly MMP-2 and -9, can be considered the prime factor in glioma invasiveness,

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since they break down the basement membrane ECM components, such as collagen type IV and laminin. MMP-2 and -9 expression also correlates with the progression and the degree of malignancy of gliomas [23,24].

SPARC is an important multifunctional glycoprotein that influences a number of biological processes, including cell differentiation, migration and proliferation, modulating cellular interactions with ECM. In particular, SPARC regulates focal cell adhesion and cell–matrix interactions, binding to structural matrix proteins, such as collagens and vitronectin. Since in the mechanisms leading to tumor invasion the ability of the tumor cell to migrate and invade the surrounding environment is dependent on the modulation of the interactions between tumor cells with ECM components, SPARC may have a functional role during tumor cell invasion into adjacent brain tissue.

With its counter-adhesive properties, SPARC affects cell–matrix interactions [25–27] and therefore may well have a functional role in tumor cell invasion of adjacent brain tissue. SPARC is also involved in proteolytic pathways by increasing the expression of collagenase and MMP-9, and activating MMP-2 [28]. This protein is frequently overexpressed in gliomas, and its expression correlates with glioma invasion *in vitro* and *in vivo* [29–31]. SPARC may be a marker of invading cells.

In this study we analyzed the effect of Ukrain on human cultured glioblastoma cells to investigate whether it affected cell proliferation, and the expression of genes and proteins involved in the mechanisms leading to tumor invasion.

Materials and methods

Cell cultures

Three human glioblastoma MI cell lines (T60, T63, GBM) were obtained from biopsy specimens, as described elsewhere [32]. Cell lines were maintained by serial passages in RPMI 1640 medium containing 10% heat-inactivated FBS at 37°C in a 5% CO_2 atmosphere and were used within the first 20 passages. Glioblastoma cells (grade IV glioma cells) were cultured in RPMI supplemented with 10% FBS and 10 U/ml penicillin plus 10 mg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Drug preparation

Ukrain (Nowicky Pharma, Vienna, Austria) was obtained as a 5-mg *C. majus* L. alkaloid thiophosphoric acid derivative plus triethylene-thiophosphoric acid triamide (Thio-TEPA)/5 ml H_2O. According to the manufacturer's instructions, the stock solution was stored at room temperature. Working solutions contained 0.1, 1 and 10 µmol/l Ukrain.

Ukrain treatment

Glioblastoma cells were initially treated with different doses of Ukrain (0.1–100 µmol/l) to establish the highest dose for cell treatment that had no cytotoxic effect. Glioblastoma cells were then treated with three final concentrations of Ukrain (0.1, 1 and 10 µmol/l). Untreated cultures served as controls. Controls and treated cells were incubated for 24, 48 and 72 h. Each cell line was cultured in duplicate. Glioblastoma cell viability was determined by Trypan blue staining.

RT-PCR analysis

Total RNA was extracted by a modification of the guanidine isothiocyanate/phenol/chloroform method (Tri-Reagent; Sigma, St Louis, Missouri, USA). After DNase I digestion, 1 µg of total RNA was reverse transcribed in 20 µl final volume of reaction mix (Promega, Milan, Italy). The following primers were used: GAPDH 5′-ATTC-CATGGCACCGTCAAGGCT, 3′-TCAGGTCCACCAC-GACACGTT (571 bp); MMP-2 5′-CCTCTCCACTG-CCTTCGATACACC, 3′-AGCATCTATTCTTGGGCA-CCG (162 bp); SPARC 5-ACCATGAGGGCCTGGATC, 3′-GGAGTGGATTTAGATCACAAG (936 bp).

Amplification reactions were conducted in a final volume of 25 µl containing 2.5 µl of cDNA, 200 µmol/l of the four dNTPs, 100 pmol of each primer, and 2.5 U of Taq DNA polymerase (EuroTaq; Euroclone, Pero, Milan, Italy). The RT-PCR protocols are listed in Table 1. The RT-PCR products were resolved by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and quantified in duplicate by densitometric analysis (Image Pro-Plus). Changes in mRNA levels are expressed as normalized optical densities relative to GAPDH mRNA.

SDS-zymography

Pro-MMP-2 and -9 protein levels were measured by SDS-zymography in the supernatants of cultured glioblastoma cells. Supernatants were concentrated in an Amicon Y10 at 6500 *g* for 15 min at 4°C. The concentrated culture media were mixed 3:1 with sample buffer containing 10% SDS; 60 µg total protein per sample was run under non-reducing/non-denaturing conditions (with SDS, but without heat denaturation of samples) onto 7.5%

Table 1 RT-PCR amplification conditions

Gene	Protocol	No. cycles
MMP-2	denaturation 94°C 1 min; annealing 60°C 2 min; elongation 72°C 3 min	30
SPARC	denaturation 94°C 1 min; annealing 55°C 1 min; elongation 72°C 1 min	32
GAPDH	denaturation 94°C 30 s; annealing 62°C 1 min; elongation 72°C 1 min + 72°C 10 min to finalize extension	25

polyacrylamide gel SDS–PAGE co-polymerized with 1 mg/ml type I gelatin. The gels were run at 4°C. After SDS–PAGE, the gels were washed twice in 2.5% Triton X-100 for 30 min each, and incubated overnight in a substrate buffer at 37°C Tris–HCl 50 mmol/l, $CaCl_2$ 5 mmol/l and NaN_3 0.02%, pH 7.5. After staining the gels with Coomassie brilliant blue R250, the MMP gelatinolytic activity was detected as clear bands on a blue background [33]. To confirm the identity of MMP gelatinolytic activity, purified MMP-1 and -2 (100 ng; Calbiochem, San Diego, California, USA) were run as controls.

Western blot

Concentrated culture media (20 µg of total proteins) were diluted in SDS-sample buffer, loaded on 10% SDS–polyacrylamide gel, separated under reducing and denaturing conditions at 80 V according to Laemmli [34], and transferred at 90 V to a nitrocellulose membrane in 0.025 mol/l Tris, 192 mmol/l glycine and 20% methanol, pH 8.3 [35]. After electroblotting, the membranes were air dried and blocked for 1 h. After being washed in TBST (TBS/Tween 20 0.05%), membranes were incubated for 1 h at room temperature in monoclonal antibody to SPARC (1:100 in TBST; Novocastra, Newcastle upon tyne, UK) and, after washing, in HRP-conjugated rabbit anti-mouse serum (1:40,000 dilution, Sigma). Immunoreactive bands were revealed using the Opti-4CN substrate (Bio-Rad, Segrate, Milan, Italy).

Statistical analysis

All tests were run in duplicate. Data from the two runs are expressed as means ± SEM and were analyzed by ANOVA followed by the Student–Neumann–Keuls test. $P < 0.05$ was considered significant.

Results

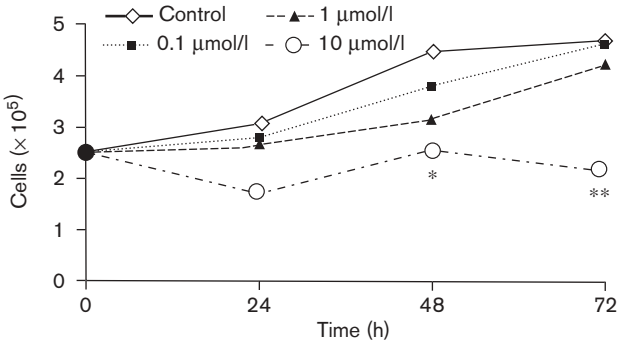
Cell viability and proliferation

The results for controls and Ukrain-treated human glioblastoma cells are presented in Fig. 1. The dose of 0.1 µmol/l Ukrain slightly affected cell proliferation. After 1 µmol/l Ukrain there was a tendency to less proliferation in treated cells, compared to controls, at all the intervals considered (reductions of 11, 26 and 14%, respectively, after 24, 48 and 72 h). After the higher dose, 10 µmol/l, proliferation was reduced at all the intervals of time, compared to controls (by 41, 41 and 45%). In particular, the high dose (10 µmol/l) significantly reduced cell proliferation after 48 h ($P < 0.05$ versus controls) and 72 h ($P < 0.05$ versus controls and 1 µmol/l Ukrain).

MMP-2 and SPARC gene expression

Figure 2(a) shows MMP-2 gene expression. A slight tendency to lower MMP-2 mRNA levels was observed in glioblastoma cells 48 and 72 h after 10 µmol/l Ukrain (10 and 5%, compared to controls, respectively). SPARC mRNA levels in controls and Ukrain-treated glioblastoma cells are shown in Fig. 2(b). Again, there was a tendency to downregulation 48 and 72 h after 10 µmol/l Ukrain (11 and 12% less than controls, respectively).

Fig. 1



Time-dependent effect of Ukrain on cultured glioblastoma cell proliferation. Cells were plated in T-25 flasks (250 000 cells/flask) and allowed to attach. Fresh medium containing 0.1, 1 and 10 µmol/l Ukrain was added, and cells were counted at the times indicated. Untreated glioblastoma cells were used as controls. Each time point represents the mean ± SEM of duplicate samples. *$P < 0.05$ versus control (48 h); **$P < 0.05$ versus control and 1 µmol/l (72 h).

SDS-zymography

SDS-zymography analysis showed two lysis bands corresponding to pro-MMP-2 and -9 (Fig. 3a). Densitometric analysis of the pro-MMP-2 band indicated a 26% decrease of pro-MMP-2 levels 72 h after 10 µmol/l Ukrain and a 17% decrease for pro-MMP-9 at the same time, compared with controls (Fig. 3b and c).

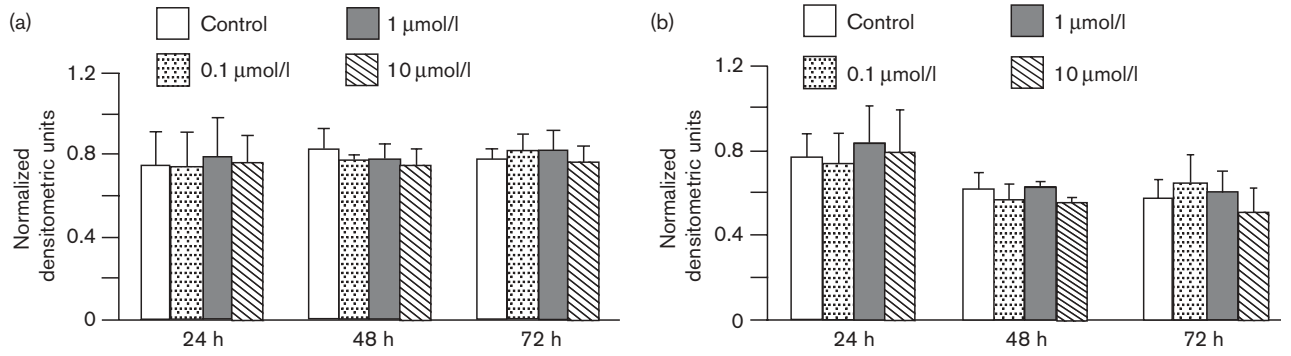
Western blot

Densitometric analysis of immunoreactive bands indicated that SPARC protein levels tended to decrease in glioblastoma cells 48 h after 10 µmol/l Ukrain (20% less than controls). At 72 h, there were dose-dependent drops in protein levels (13, 22 and 30%, respectively, after 0.1, 1 and 10 µmol/l Ukrain, compared with controls; P ANOVA = 0.002) (Fig. 4a and b).

Discussion

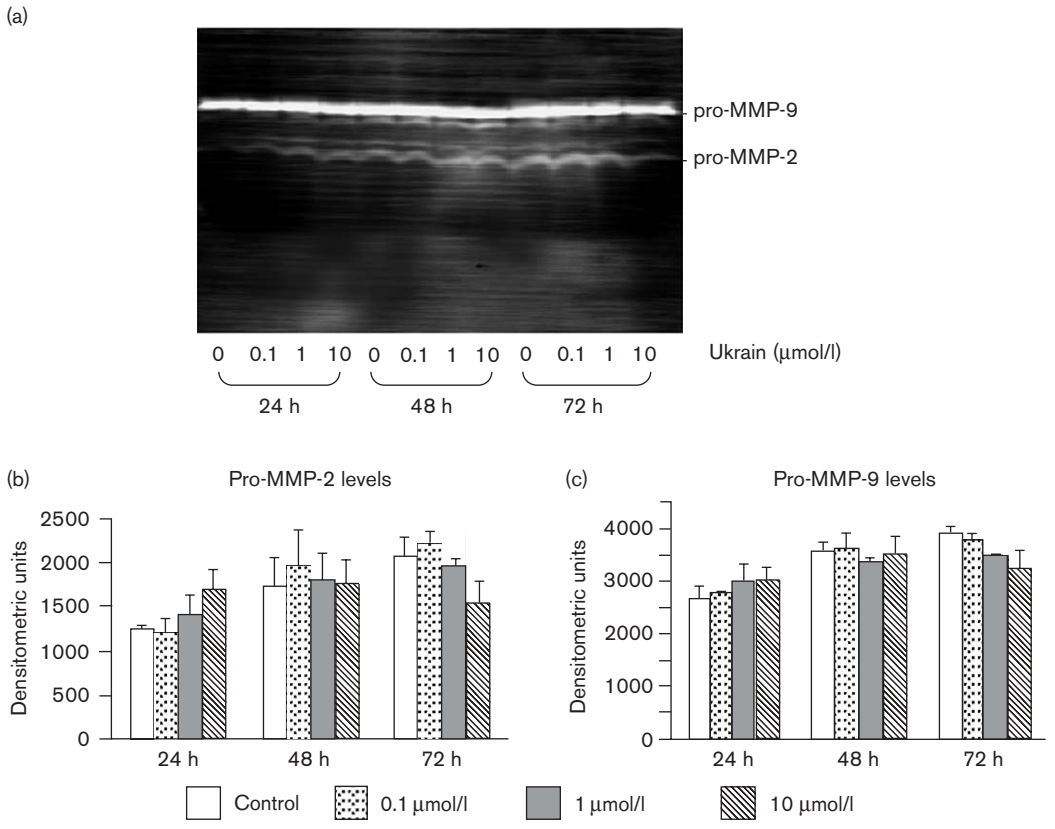
The highly invasive phenotype of malignant gliomas means that the prognosis is poor, even using multidisciplinary approaches combining surgery, radiotherapy and chemotherapy [11]. Therefore, a better therapeutic strategy is still awaited for these brain tumors. In the search for new anti-tumoral agents over the past years, many plant extracts have been investigated. One is Ukrain, derived from the celandine plant. This is an anticancer compound with activity against a range of solid tumors, including those in the colon, rectum, breast, pancreas, bladder and ovary [6–10].

Fig. 2



Bar graphs showing steady-state mRNA levels of MMP-2 (a) and SPARC (b) in cultured human glioblastoma cells at the times indicated. Changes in mRNA levels are expressed as normalized optical densities relative to GAPDH mRNA. Means ± SEM.

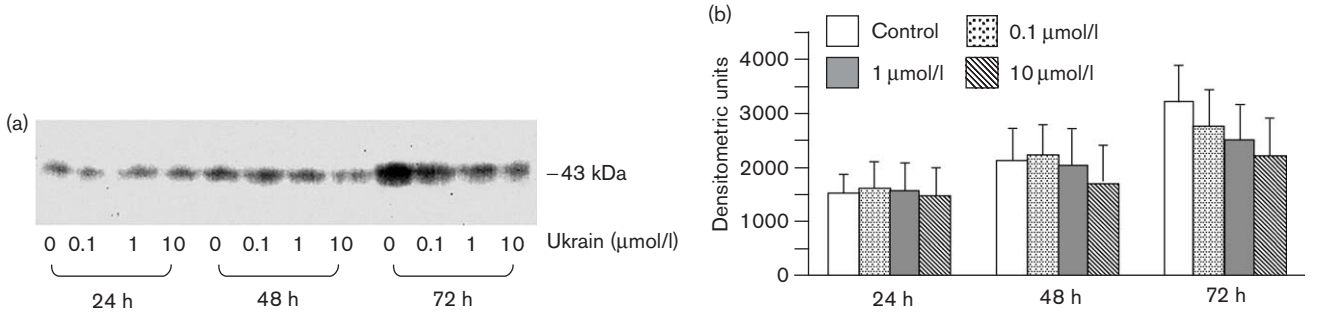
Fig. 3



(a) Representative SDS-zymography showing two lysis bands consistent with pro-MMP-2 and -9. Glioblastoma supernatants were electrophoresed on 7.5% gels. (b) Bar graphs showing pro-MMP-2 and (c) -9 levels after densitometric scanning of lysis bands. Means ± SEM.

We employed three doses of Ukrain to investigate *in vitro* whether this drug has some effect on glioblastoma cell proliferation and on the expression of genes involved in the mechanisms leading to tumor invasion. Ukrain reduced glioblastoma cell proliferation 24, 48 and 72 h after treatment. This pattern was dose dependent and

Fig. 4



(a) Western blot analysis for SPARC protein in glioblastoma supernatants. The monoclonal antibody identifies a positive immunoreactive band in the 43-kDa region corresponding to SPARC. (b) Bar graphs showing SPARC protein levels after densitometric scanning of immunoreactive bands. Means ± SEM.

proliferation appeared already to be affected by 1 µmol/l Ukrain, which lowered cell proliferation compared with controls. This effect was more evident after the 10 µmol/l dose, which elicited a significant decrease of cell proliferation. Interestingly, the proliferation curve of glioblastoma cells treated with 10 µmol/l Ukrain indicated that the number of cells remained the same at all times, compared to untreated cells, pointing to complete inhibition of cell proliferation and suggesting that the high dose had a cytostatic effect.

In accordance with previous findings, we speculate that the concentration-dependent effect of Ukrain on tumor cell viability very likely involves inhibition of DNA, RNA and protein synthesis [36], and possibly, as previously reported, also by an effect on the induction of apoptosis [37,38].

The expression of genes and proteins involved in the ECM remodeling associated with tumor invasion showed that the two main determinants, MMP and SPARC, were differently targeted by Ukrain. MMP-2 and -9 are the gelatinases mainly involved in ECM remodeling, allowing tumor cells to break down basement membranes and diffuse into the surrounding tissues. Their expression also correlates with the progression and the degree of malignancy of gliomas [23,24].

Ukrain had no effect on MMP-2 mRNA levels. However, pro-MMP-2 and -9 showed a tendency to lower protein levels 72 h after the highest dose of the drug. These results suggest that the high dose of Ukrain slightly affects gelatinase expression at the protein level, although the difference between Ukrain-treated and control samples was not statistically meaningful.

SPARC is a matricellular protein required for interactions between cells and their extracellular environment and, on account of its counter-adhesive properties, it may have a functional role in tumor cell invasion of adjacent brain tissue. Since this protein is frequently overexpressed in gliomas, and its expression correlates with glioma invasion *in vitro* and *in vivo* [29,30], SPARC may be considered a marker of invading cells. This protein may possibly play a major role in the promotion of glioma cell invasion, as confirmed by evidence that human glioma cells engineered to overexpress SPARC adopt an invasive phenotype [31].

Another role for SPARC in the promotion of tumor progression has also recently been suggested [39] – it may facilitate tumor cell survival under the stressful conditions that surround the tumor, such as nutrient restriction, hypoxia and genomic instability. The expression of SPARC by gliomas induces cellular survival in serum-free conditions and the apoptotic rate of SPARC-expressing glioma cell lines is reduced compared to a control line. This might be a mechanism through which gliomas resist cell death [16]. SPARC, therefore, being involved in tumor invasion and resistance to apoptosis, may be an important target for the design of strategies for cancer therapy, especially to restrict glioma invasion.

Ukrain affects SPARC expression. SPARC mRNA levels were slightly reduced by the high dose, whereas at the protein level the drug had an evident dose-dependent effect, leading to downregulation of SPARC protein expression, although the difference between Ukrain-treated and control samples was not significant. However, since patients receiving pharmacological anti-tumor therapy receive chronic treatment, protracted treatment with Ukrain might well have worthwhile activity against glioblastoma.

As a whole, our results suggest that Ukrain influences some major aspects of progression in human glioblastoma

cells, such as cell proliferation and the expression of a pivotal protein in the mechanisms, leading to tumor cell invasion and survival. Thus, Ukrain may have some potential for the therapy of brain tumors, and could well also help extend our understanding of the mechanisms of this anti-tumor and chemopreventive potential.

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