Ukrain modulates glial fibrillary acidic protein, but not connexin 43 expression, and induces apoptosis in human cultured glioblastoma cells

Nicoletta Gagliano[a], Claudia Moscheni[a], Carlo Torri[a], Elena Donetti[a], Ivana Magnani[b], Francesco Costa[a], Wassil Nowicky[c] and Magda Gioia[a]

Glioblastoma is a highly malignant tumor, characterized by an unfavorable prognosis even in response to multidisciplinary treatment strategies, owing to its high-invasive phenotype. Ukrain, a semisynthetic 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. In particular, we previously demonstrated that Ukrain modulates the expression of genes and proteins involved in tumor invasion, and here we investigate some unreported effects of Ukrain on human cultured glioblastoma cells. We used morphological and molecular biology methods to analyze the expression and the intracellular distribution pattern of glial fibrillary acidic protein, the expression of the gap junction protein connexin 43 and the apoptotic effect in human glioblastoma cells treated with 0.1, 1 and 10 μmol/l Ukrain for 72 h. After treatment with 10 μmol/l Ukrain, glial fibrillary acidic protein fluorescence increased and a higher number of cells displayed glial fibrillary acidic protein organized into a filamentous state. Western blot analysis of glial fibrillary acidic protein confirmed that Ukrain tended to upregulate the protein. Connexin 43 was not modulated by Ukrain both at the mRNA and at the protein level. Ukrain-induced apoptotic rate was 4.63, 10.9 and 28.9% after 0.1, 1 and 10 μmol/l Ukrain, respectively, likely mediated by cytochrome *c* release in the cytoplasm. Considered as a whole, these findings provide new information to complete the understanding of the mechanisms of Ukrain antitumor and chemopreventive effect, and support the possible potential of Ukrain for the therapy of brain tumors.
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[a]Department of Human Morphology, [b]University of Milan and Division of Medical Genetics, San Paolo School of Medicine, University of Milan, Italy and [c]Ukrainian Anti-Cancer Institute, Vienna, Austria

Correspondence to Dr Nicoletta Gagliano, PhD, Department of Human Morphology, University of Milan, Via Fratelli Cervi 93, 20090 Segrate, Milano, Italy
Tel: +39 02 50330462; fax: +39 02 50330452;
e-mail: nicoletta.gagliano@unimi.it

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Introduction

Gliomas are the most common primary brain tumors, accounting for more than 40% of all central nervous system neoplasms [1]. Among gliomas derived from neoplastic transformation of astrocytes, four major grades are defined, glioblastoma (IV grade astrocytoma) being a highly malignant tumor typically affecting adults between 45 and 60 years of age [2]. Neoplastic astrocytes may be identified by the expression of the intermediate filament protein, glial fibrillary acidic protein (GFAP), a cell type-specific marker for astroglial cells. With increasing astrocytic malignancy, there is a progressive loss of GFAP production [3]. GFAP protein comprises an amino-terminal head domain, a central rod domain, and a carboxy-terminal head domain [4]. GFAP can be assembled or disassembled depending on its phosphorylation status, thus affecting the cytoskeletal network, and therefore influencing astrocytoma cell morphology, motility, differentiation, growth and mitosis.

Gap junctions are an important means for intercellular communication during development, tissue differentiation and maintenance of homeostasis, allowing the coordinated responses of adjacent groups of cells to external stimuli [5,6]. Gap junctions are channels made up by the association of two half-channels (connexons), each connexon being homomultimer of six connexins (CX), among which CX43 is the most ubiquitously expressed and abundant gap junction protein in the central nervous system, and is primarily expressed in astrocytes, as also in glioma cells [7].

Expression of CX43 is often decreased in human tumor cells and tissues, including glioblastoma, which, frequently, lacks functional gap junctions [8] and the growth of this highly malignant tumor is inversely correlated with the expression of these junctions. Besides their quantity, the gap junction functional status also plays a key role. In fact, the functional status of connexon is also modulated

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by posttranslational modification via phosphorylation of CX43. Loss of intercellular gap junctions is a distinctive morphological feature of apoptotic cells.

Apoptosis is a highly regulated form of cell death involving complex intracellular proteolysis in which the main executors are caspases. The apoptotic mechanism may be triggered either by the extrinsic death receptor-dependent or by the intrinsic mitochondrial pathway. The latter is characterized by the disruption of the mitochondrial integrity with the release of proapoptotic proteins, including cytochrome *c*, from the mitochondrial intermembrane space into the cytosol, culminating in the activation of caspase-9 that finally executes cell death [9].

The anticancer drug Ukrain (NSC 631570) contains alkaloids of greater celandine (*Chelidonium majus*, a member of the Papaveraceae family) [10], made water soluble by derivatization with thiotepa, displaying a putative proapoptotic effect.

Some clinical investigations suggested that Ukrain, given as single drug or in combination with conventional chemotherapy, exerts beneficial effects in the treatment of a range of solid tumors, including the colon, rectum, breast, pancreas, bladder and ovary [11–15].

The molecular mechanisms of Ukrain-induced antineoplastic effects, however, are not yet completely understood, although an immunostimulating and immunomodulating [16], antiangiogenic on human endothelial cells *in vitro*, and antiapoptotic actions [17–19] of Ukrain have been reported.

In our previous study, we demonstrated the effect of Ukrain on cell proliferation, and on the expression of genes and proteins involved in the key mechanisms of tumor invasion in cultured human glioblastoma cells [20], suggesting that this drug may have a possible role for the glioblastoma treatment.

Up to now, however, the possible effects of Ukrain on tumors, and in particular on glioblastoma, are not fully defined, therefore in this study we aimed at investigating in human cultured glioblastoma cells some different major aspects characterizing malignant tumors and possibly targeted by the anticancer action of Ukrain.

Here, we analyzed the expression and intracellular distribution of GFAP, expression of the gap junction protein CX43 and the apoptotic effect in human glioblastoma cells treated with Ukrain.

Materials and methods

Cell cultures

Three human glioblastoma MI cell lines (T60, T63 and glioblastoma) were obtained from biopsy specimens, as described elsewhere [21]. All procedures are in accordance with the Helsinki declaration. Cell lines were maintained by serial passages in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO_2 atmosphere and were used within the first 20 passages. Glioblastoma cells (grade IV glioma) were cultured in RPMI supplemented with 10% fetal bovine serum, 10 U/ml penicillin and 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 water soluble *C. majus* alkaloid thiophosphoric acid derivative in 5 ml water for injection. 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 then treated with three final concentrations of Ukrain (0.1, 1 and 10 µmol/l). Untreated cultures served as controls (CT). CT and treated cells were incubated for 72 h. Each cell line was cultured in duplicate. Glioblastoma cell viability was determined by Trypan blue staining.

Transmission electron microscopy

Cells were harvested by centrifugation at 100*g* for 10 min and therefore fixed in 3% glutharaldehyde in 0.1 mol/l pH 7.4 phosphate buffer (overnight at room temperature); after washing, cells were postfixed in 1% osmium tetroxide in 0.1 mol/l pH 7.4 phosphate buffer (60 min at 4°C), washed by distilled water at 4°C, dehydrated through an ascending series of acetone and embedded in araldite. Ultrathin sections (80 nm) were sliced with a Reichert Ultracut R ultramicrotome (Leica, Vienna, Austria), stained with uranyl acetate and lead citrate, and then observed with a JEOL CX100 transmission electron microscope (JEOL, Tokyo, Japan).

Immunocytochemistry

Glioblastoma cells were cultured on round coverslips in multi-wells. When they were at subconfluence, cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 10 min, postfixed in 70% ethanol and stored at −20°C until use. The cells were then washed in PBS three times and incubated overnight at 4°C with monoclonal anti-GFAP primary antibody (1 : 800; Chemicon, Temecula, California, USA) or monoclonal anti-cytochrome *c* (1 : 100; Santa Cruz Biotechnology, Santa Cruz, California, USA). Secondary antibody conjugated with Alexa 488 (1 : 500; Molecular Probes, Invitrogen, Italy) was applied for 1 h at room temperature, followed by rinsing with PBS. Negative CT sections were incubated omitting the primary antibody. After the labeling procedure was completed, the cover-

slips were mounted onto glass slides using a mounting medium with 4′,6-diamidino-2-phenylindole. The cells were photographed by a digital camera connected to the microscope (Nikon, Calenzano, Florence, Italy).

Annexin V assay

Apoptosis was assayed by the detection of membrane externalization of phosphatidylserine with Annexin V–fluorescein isothiocyanate conjugate, using an Annexin V assay kit (Calbiochem, Milan, Italy) according to manufacturer's instructions. Apoptosis was detected by the appearance of patches of fluorescence on the cell surface.

Reverse transcription-polymerase chain reaction analysis

Total RNA was extracted by a modification of the guanidine isothiocyanate/phenol/chloroform method (Tri-Reagent; Sigma, Milan, Italy). After DNase I digestion, 1 μg of total RNA was reverse-transcribed in 20 μl final volume of reaction mix (Promega Italia, Milan, Italy). The following primers were used: glyceraldehyde-3-phosphate dehydrogenase 5′-ATTCCATGGCACCGTCAAGGCT, 3′-TCAGGTCCACCACGACACGTT (571 bp, 25 cycles); CX43 5′-GGACATGCACTTGAAGCAGA, 3′-GGTCGCTCTTTCCCTTAACC (368 bp, 30 cycles).

Amplification reactions were conducted in a final volume of 25 μl containing 2.5 μl of cDNA, 200 μmol/l of the four deoxyribonucleotide triphosphates, 100 pmol of each primer and 2.5 U of Taq DNA polymerase (EuroTaq, Euroclone, Milan, Italy). The reverse transcription-polymerase chain reaction products were resolved by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and quantified in duplicate by densitometric analysis (UVSave; Eppendorf, Milan, Italy). Changes in mRNA levels are expressed as normalized optical densities relative to glyceraldehyde-3-phosphate dehydrogenase mRNA.

Western blot

Cells were lysated in Tris–HCl 50 mmol/l pH 7.6, 150 mmol/l NaCl, 1% Triton X-100, 5 mmol/l ethylenediaminetetraacetic acid, 150 mmol/l $MgCl_2$, 1% sodium dodecyl sulfate (SDS) and proteases inhibitors. Thirty micrograms of total proteins were diluted in SDS-sample buffer, loaded on 10% SDS–polyacrylamide gels, separated under reducing and denaturing conditions at 80 V according to Laemmli [22], and transferred at 90 V to a nitrocellulose membrane in 0.025 mol/l Tris, 192 mmol/l glycine, 20% methanol, pH 8.3 [23]. After electroblotting, the membranes were air dried and blocked for 1 h. After being washed in TBST (Tris-buffered saline/Tween-20, 0.05%), membranes were incubated for 1 h at room temperature in monoclonal antibody to GFAP (1 : 500 in TBST; Santa Cruz Biotechnology) and, after washing, in horseradish peroxidase-conjugated rabbit anti-mouse serum (1 : 40 000; Sigma). To confirm equal loading, SDS polyacrylamide gels were stained by Coomassie blue. To investigate CX43 protein levels, membranes reacted with polyclonal anti-CX43 (2 μg/ml in TBST; Zymed, Invitrogen) and horseradish peroxidase-conjugated anti-rabbit serum (1 : 20 000; Sigma). To confirm equal loading, membranes were reprobed by monoclonal antibody to α-tubulin (1 : 2000; Sigma). Immunoreactive bands were revealed using the Opti-4CN substrate (Bio-Rad, Milan, Italy).

Statistical analysis

All tests were run in duplicate. The GraphPad Prism version 3.0 software package (GraphPad Software, San Diego, California, USA) was used for the statistical analysis. Data are expressed as mean ± standard error and were analyzed by one-way analysis of variance followed by the Student–Neumann–Keuls test. P values less than 0.05 were considered significant.

Results

Glial fibrillary acidic protein expression

When analyzed for GFAP expression by immunofluorescence, cells of all three lines exhibited GFAP immunofluorescence with variations in intensity, evident both in Ukrain-untreated and Ukrain-treated cells (Fig. 1). In general, most of glioblastoma cells, independently on Ukrain treatment, showed only a diffuse and weak fluorescent signal for GFAP, revealing a wide range of expression. The CT sections which had been incubated omitting the primary antibody showed no labeling. Interestingly, GFAP immunofluorescence seemed more intense in cells with normal nuclei, whereas it was weaker or undetectable in cells with small nuclei, showing a tendency to nuclear condensation and fragmentation, typical features of early phases of apoptosis. Moreover, after treatment with 10 μmol/l Ukrain, GFAP fluorescence increased and a higher number of cells displayed GFAP organized into a filamentous state.

Western blot analysis of GFAP expression revealed that Ukrain induced an upregulation of the protein, although without significant differences in all three cultures (Fig. 2).

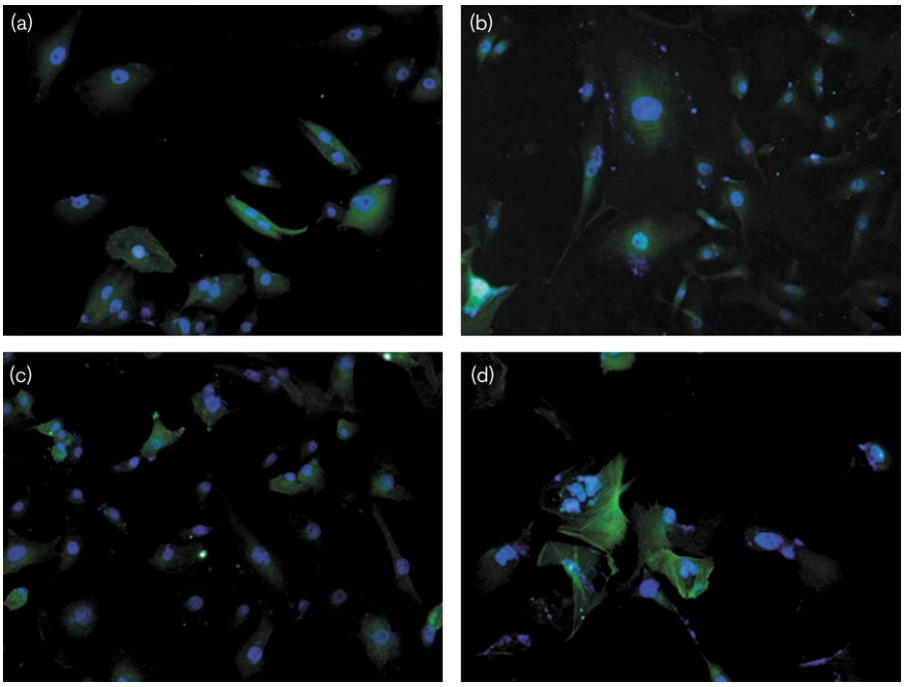
Connexin 43 expression

CX43 expression was evaluated both at the mRNA and at the protein level. Gene expression analysis shows that CX43 is not modulated by Ukrain at the mRNA level (Fig. 3a). The same pattern was observed for the CX43 protein analysis (Fig. 3b).

Apoptosis

The apoptotic process was analyzed by three different approaches, which are Annexin V assay, analysis of

Fig. 1



Microphotographs showing fluorescent labeling for glial fibrillary acidic protein in controls (a), and 0.1 (b), 1 (c) and 10 μmol/l (d) Ukrain-treated cells (T60). Original magnification, × 20.

cytochrome *c* distribution and by transmission electron microscopy (TEM).

The Annexin V assay detects the exposed phosphatidylserine during apoptosis. The quantification of the number of apoptotic cells showed that Ukrain treatment induced apoptosis in glioblastoma cells. The mean apoptotic rate was 4.63, 10.9, and 28.9% after 0.1, 1 and 10 μmol/l Ukrain, respectively ($P < 0.05$ for 10 μmol/l Ukrain vs CT, 0.1 and 1 μmol/l Ukrain) (Fig. 4). The observation of fluorescence revealed that only few cells were stained with propidium iodide, suggesting that few Ukrain-treated cells are neither in a late phase of apoptosis nor necrotic.

These data are consistent with the data obtained by the TEM observations. In fact, TEM analysis did not reveal apoptotic bodies and cellular debris. In 10 μmol/l Ukrain-treated glioblastoma cells, a slight chromatin condensation was observed, supporting the occurrence of very early phases of apoptosis (Fig. 5). Ukrain also induced the release of cytochrome *c* in the cytosol after 10 μmol/l Ukrain treatment, whereas in untreated, and 0.1 and 1 μmol/l Ukrain-treated cells the cytochrome *c* labeling gave a punctate staining as observed in nonapoptotic cells (Fig. 6).

Discussion

Glioblastoma cells are characterized by cellular heterogeneity [24] and, because of their exceptional migratory capacity, they are able to diffusely infiltrate normal brain [25], making total surgical removal impossible; therefore, patients have a poor prognosis, even in response to multidisciplinary treatment strategies including surgery, radiotherapy and chemotherapy [26,27].

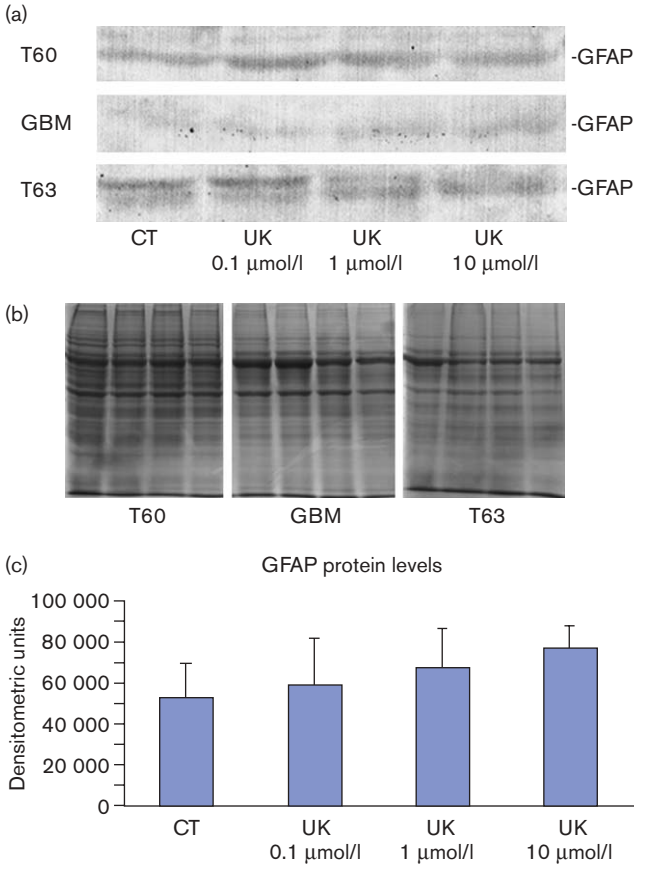
As a consequence, it is important to explore new therapeutic paradigms and a better therapeutic strategy is still awaited for these brain tumors.

Ukrain was previously demonstrated to influence 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 [20], and here we describe some unreported effects of this drug on human cultured glioblastoma cells.

Glial fibrillary acidic protein

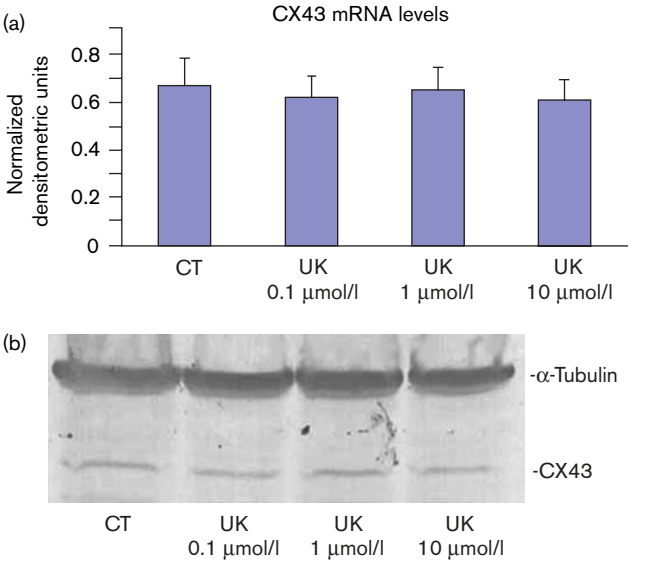
GFAP is the intermediate filament protein marker of astroglial cells, whose assembly/disassembly status is regulated by phosphorylation, thus affecting the cytoskeletal network [28]. GFAP is also present in astroglial cells nuclei as the major component of the nuclear lamina.

Fig. 2



(a) Representative Western blot analysis for glial fibrillary acidic protein (GFAP) protein in T60, GBM (glioblastoma) and T63 glioblastoma cells. The monoclonal antibody identifies a positive immunoreactive band in the 50-kDa region corresponding to GFAP. (b) Parallel sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels were stained by Coomassie blue to confirm equal loading. (c) Bar graphs showing GFAP protein levels after densitometric scanning of immunoreactive bands. Mean ± SEM. CT, control; UK, Ukrain.

Fig. 3

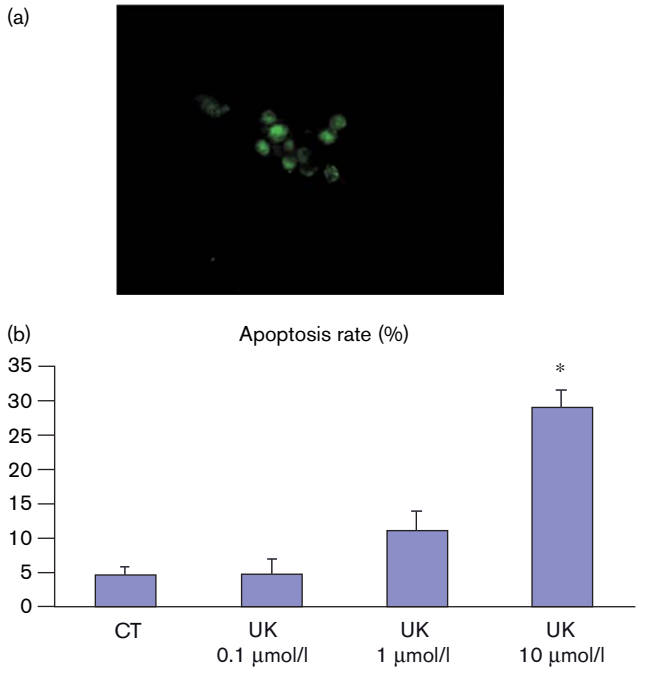


(a) Bar graphs showing steady-state mRNA levels of connexin 43 (CX43) in cultured glioblastoma (GBM), T60 and T63 human glioblastoma cells at the times indicated. Changes in mRNA levels are expressed as normalized optical densities relative to glyceraldehyde-3-phosphate dehydrogenase mRNA. Mean ± SEM. (b) Representative Western blot analysis of CX43 protein in GBM glioblastoma cells. The monoclonal antibody identifies positive immunoreactive bands corresponding to native unphosphorylated (P0) species of CX43. To confirm equal loading, membranes were reprobed by monoclonal antibody to α-tubulin, as described in the Materials and methods section. CT, control; UK, Ukrain.

Most glioblastoma cells exhibited GFAP immunofluorescence with variations in intensity, and, in general, they showed only a diffuse and weak fluorescent signal for GFAP. This is consistent with the previously reported reduced GFAP-positivity in high-grade astrocytomas [29]. We, however, found a dose-dependent tendency to GFAP increase in Ukrain-treated glioblastoma cells, as shown by Western blot analysis. This is an interesting finding, as a progressive loss of GFAP production is described with increasing astrocytic malignancy [4]. Moreover, a relationship between increased GFAP protein expression and suppression of glial tumor growth [30], inhibition of cell-cycle progression, and decreased proliferation was previously reported [31]. The increased expression of GFAP in human glioblastoma cells was also related to reduced cell motility [32], maybe as a consequence of the interactions of this intermediate filament protein with actin and other cytoskeletal proteins.

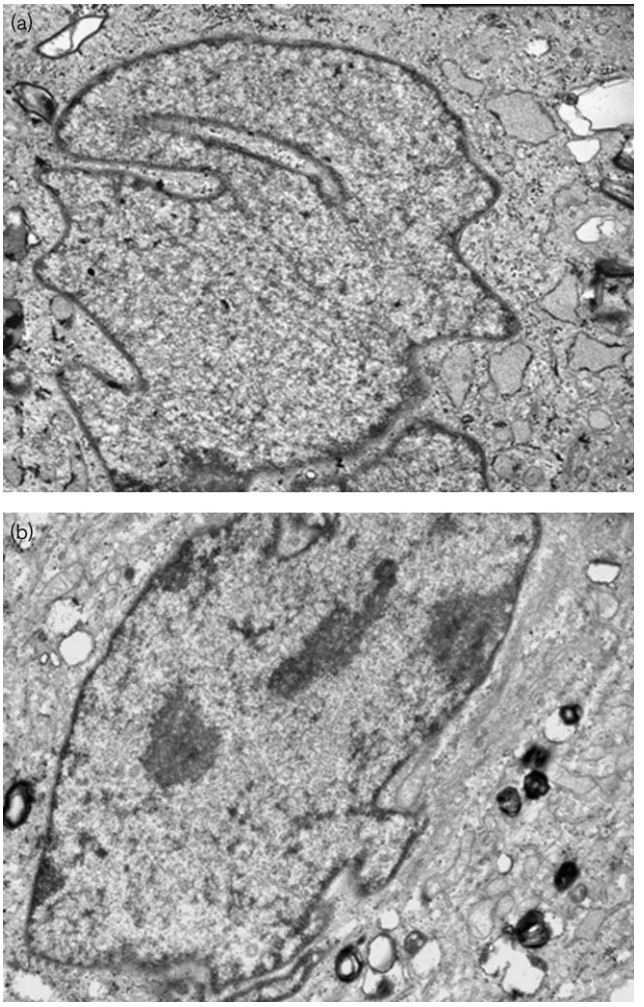
Our results show that Ukrain affected GFAP also at the morphological level. In fact, we observed a different immunofluorescence intensity in relation to nuclear morphology and a different organization of GFAP in Ukrain-treated cells, in which the protein seemed arranged in a filamentous state in a higher number of cells, compared with CT, in particular after 10 μmol/l of Ukrain. These observations suggest two considerations. First, GFAP is thought to stabilize glial cell cytoskeleton through the interactions between GFAP filaments, the nuclear membrane and the plasma membrane [33]. Interestingly, following Ukrain administration this structural link between the nucleus and the plasma membrane seemed less present in cells displaying the nuclear morphology typical of apoptosis. As a consequence, Ukrain may reduce glioblastoma cells' motility, possibly influencing their invasive potential. Second, the disassembly of the filamentous GFAP may facilitate equal separation of cytoplasmic components into two daughter cells during cytokinesis [4]. As Ukrain seemed to favor the filamentous state of the protein, the drug may inhibit cell-cycle progression and mitosis of glioblastoma cells, and therefore this may represent a possible molecular mechanism responsible for the decreased proliferation of Ukrain-treated glioblastoma cells previously described [20].

Fig. 4



(a) Representative apoptotic cells determined by Annexin V assay. Flourescent-labeled Annexin V binds to the cell membrane. This pattern, characterized by the absence of blebs, suggests that Ukrain (UK)-treated cells are in the early phases of apoptosis. Original magnification, × 20. (b) Bar graphs showing apoptosis rates in glioblastoma (GBM), T60 and T63 glioblastoma cells. Mean ± SEM. *$P < 0.05$ for 10 vs. 0.1 μm Ukrain. CT, control.

Fig. 5



Representative electron photomicrographs of ultrathin sections of glioblastoma cells (T60) showing nuclear ultrastructure. (a) Untreated cells (original magnification, × 10 000). (b) Glioblastoma cells after 10 μmol/l of Ukrain, showing nuclear morphology changes after drug administration (original magnification, × 7200).

Connexin 43

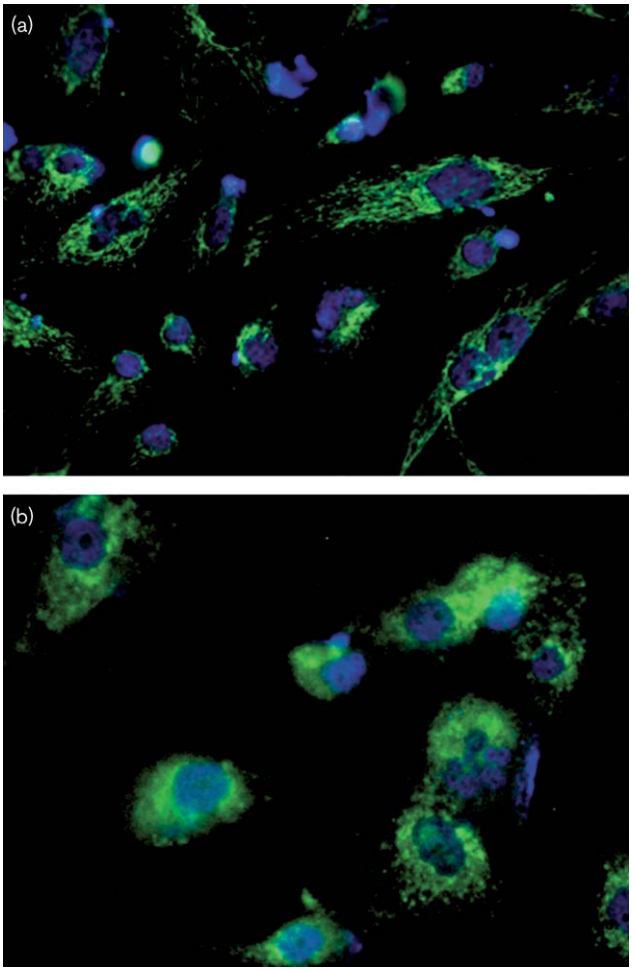
Gap junctions are to a large extent involved in the regulation of major cell functions including proliferation, differentiation and, more in general, cell homeostasis [5].

CX43 is the most abundant gap junction CX expressed in glioma cells and a reduced expression of CX43 has been demonstrated in numerous cancer cells [7], including glioblastoma. In fact, in glioblastoma biopsies, a decreased gap junction intercellular communication (GJIC) corresponds to decreased expression of CX43 and increased cellular proliferation [34].

Our findings show that CX43 expression is not modulated by Ukrain at the mRNA level, as previously shown [31]. The functional state of the CX is also modulated by phosphorylation and our results show that the nonphosphorylated form of CX43 (open gap junctions) is not modified by Ukrain, thus suggesting that the drug does not affect CX functional state [35].

As gap junctions are closely associated with the CT of cell growth [6], growing evidence suggests that CX43 may function as a tumor suppressor gene [5] and therefore may be involved in the promotion of apoptosis. One of the key cell components to maintain homeostasis is GJIC and one of the mechanisms responsible for the maintenance of cell homeostasis by GJIC may involve the regulation of apoptosis [5]. The role of GJIC and CX43 in the regulation of apoptosis is supported by the evidence that tumor promoters, which inhibit apoptosis, can block GJIC, whereas tumor-suppressing agents, which may augment apoptosis, enhance GJIC [36,37]. Moreover, in CX43-transfected cells the expression of CX43 led to the downregulation of bcl-2, which potentiated the apoptotic process [37,38]. Ukrain effect seems not to be involved in the transcriptional and/or posttranslational regulation of CX43 gene expression.

Fig. 6



Microphotographs showing immunofluorescence detection of cytochrome *c* in controls (a) and 10 μmol/l Ukrain-treated glioblastoma (GBM) cells (b). Untreated control cells showed a punctate cytoplasmic staining pattern typical for mitochondrial localization. Original magnification, × 20.

Apoptosis

In our previous study, we demonstrated that Ukrain dose-dependently reduced glioblastoma cell proliferation 24, 48 and 72 h after treatment [20]. In particular, the proliferation curve of glioblastoma cells treated with 10 μmol/l Ukrain indicated a complete inhibition of cell proliferation, thus suggesting a cytostatic effect likely owing to the induction of apoptosis. Here, we analyzed whether Ukrain exerted an apoptotic effect in the same experimental model 72 h after treatment.

One of the biochemical features of apoptosis is the exposure of phosphatidylserine to the outer leaflet of the plasma membrane [39]. This is an early event of the apoptotic process, which can be detected by the strong specific interaction of Annexin V to the exposed phosphatidylserine. As shown by the Annexin V assay, Ukrain induced apoptosis of glioblastoma cells.

The immunofluorescence analysis of cytochrome *c* distribution revealed that Ukrain triggers mitochondrial damage with the release of cytochrome *c*, an important molecule in the apoptosis pathway, from the mitochondrial intermembrane space into the cytosol. As a consequence, as also demonstrated in a Jurkat T-lymphoma cell model [19], induction of apoptosis contributes to the antineoplastic cytotoxic effects of Ukrain on glioblastoma cells, very likely via a pathway involving mitochondrial damage.

Conclusions

Considered as a whole, our results suggest that Ukrain affects several important functions of glioblastoma cells. Here, we demonstrate its ability to upregulate GFAP protein expression, but not CX43, and to strongly induce apoptosis. The difference between Ukrain-treated and CT samples concerning GFAP was not significant, possibly owing to the highly polymorphic behavior of glioblastoma cells.

These findings help to extend our understanding of the mechanisms of Ukrain antitumor and chemopreventive effect, and support the possible potential of Ukrain for the therapy of brain tumors.

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