Amino Acids (2005) 28: 213–219
DOI 10.1007/s00726-005-0171-3

Amino Acids
Printed in Austria

Expression of ABC-1 transporter is elevated in human glioma cells under irradiation and temozolomide treatment

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Received July 1, 2004
Accepted August 1, 2004
Published online February 23, 2005;

Summary. *Objective*: Chemo-therapeutic treatment of glioma patients has minor success. Little is known about mechanisms of a pronounced resistance of gliomas towards actual therapies, yet. ABC-1 belongs to the group of transporters known to be involved in the export of hydrophobic substances and vascular regulation. This study investigates an effect of both temozolomide (TMZ) treatment and/or irradiation on the expression of the ABC-1 transporter in human U87-MG glioma cells.

Material and methods: In parallel experiments U87-MG cells underwent either irradiation (RT), chemo-treatment (CT) using TMZ, and combined chemo/radiation-treatment (CT/RT). After each treatment the cells were incubated either 2 or 24 hours at 37°C and counted before protein analysis using Western-Blot technique.

Results and conclusions: An exponential growth of cellular density was observed for both untreated and irradiated cells being, however, about 2-times slower in irradiated compared to untreated cells. In contrast the density increase of chemo-treated cells as well as that of cells, which underwent the combined CT/RT treatment was of linear nature. ABC-1 expression was detected in untreated as well as treated cells. Increasing cell density and all kinds of treatment resulted in a considerably enhanced ABC-1 expression. CT treatment resulted in highly up-regulated ABC-1 expression especially in non-confluent cultures compared to untreated cells. Irradiation had a comparable or even higher inducible effect on the ABC-1 expression rates depending, however, on cell density. The highest expression rates were observed in cultures with high cellular density 2 hours after application of the combined treatment. Strong up-regulation of ABC-1 expression under both irradiation and chemo-treatment might be a clue to multidrug and irradiation cross-resistance mechanisms of malignant glioma cells converting the ABC-1 transporter into an attractive pharmacological target for a clinical breakthrough in the therapy of malignant gliomas.

Keywords: Glioma – Multidrug resistance – ABC-1 – Differential gene expression – Irradiation – Temozolomide treatment

Introduction

Gliomas are the most common brain tumours, and grade IV glioblastomas are almost universally fatal (Hirose et al., 2001). The poor prognosis of glioma patients is partially based on the minor success obtained from chemo-therapeutic treatment (Spiegl-Kreinecker et al., 2002). Drug transport in the central nervous system is highly regulated not only by the blood-brain and blood-cerebrospinal fluid barrier but also in brain parenchyma. Recent discovery of drug transporters localised in brain parenchyma cells, such as microglia and astrocytes, suggest a reconsideration of the present conceptualisation of the brain barrier as it relates to drug transport (Lee et al., 2001). Additionally to the blood-brain barrier, drug export at the tumour cell level is supposed to be involved in the intrinsic chemo-resistance of brain tumours. Simultaneous resistance of malignant cells to several anti-neoplastic agents that are structurally and functionally unrelated is known as multidrug resistance (MDR). The MDR protein family belongs to the ATP-binding cassette super-family (ABC) of transporters, which are ATP-driven export pumps that mediate the export of organic anions from cells. So far only little information is available on expression and physiological functions of MDR proteins in brain (Hirrlinger et al., 2002). Recently a novel brain multidrug resistance protein was identified in porcine brain capillary endothelial cells; this protein may play an important role in regulation of the blood-brain barrier and exclusion of xenobiotics from the brain (Eisenblatter and Galla, 2002). ABC transporters that have a dual function being involved in both cellular drug export and blood-brain barrier regulation may be the most attractive potential molecular targets in the improvement of an application of anticancer drug therapy in glioma patients.

At least 8 members of the ABC transporter family are known to be involved in the export of anticancer drugs, and some appear to contribute to the resistance of cancer

cells to chemo-therapy (Gottesman and Ambudkar, 2001). Furthermore, the ABC-1 exporter was recently shown to be important for the regulation of endothelial and, therefore, arterial wall functions (Bisoendial et al., 2003). The ABC-1 gene has been mapped to human chromosome 9q31 and encodes a 220-kDa glycoprotein required for engulfment of cells undergoing programmed cell death (Luciani et al., 1994; Rust et al., 1998; Becq et al., 1997). ABC-1 generates an anion flux sensitive to glibenclamide, sulfobromophthalein, and blockers of anion transporters. This anion flux is up-regulated by orthovanadate, cAMP, protein kinase A, and okadaic acid (Becq et al., 1997).

Although little is known about the multidrug resistance of gliomas, recent experiments with rat glial cell subtypes have shown that for these types of cells differential expression of drug resistance genes and chemo-sensitivity correlate with differential response of oligodendrogliomas and astrocytomas to chemo-therapy (Nutt et al., 2000). Even less is known about mechanisms of a cross-resistance of gliomas towards chemo-therapeutic agents and irradiation. There is a growing body of evidence that transcription rates of ABC-1 are dependent on DNA damage as well as the expression level of the p53 gene that is considered as one of the drug resistances genes (Nutt et al., 2000), and is regulated in response to the activity of oxidative stress factors such as NF-kappa B (Muller, 2000). Therefore, we hypothesised that the function of ABC-1 under therapeutic conditions may be associated with the cross-resistance of the treated malignant human glioma cells towards both irradiation and chemo-treatment. In this work we investigated a possible inducible effect of both irradiation and chemo-treatment on the expression level of ABC-1 in glioma cells.

Material and methods

Cell preparation and treatment

Human U87-MG glioma cells were cultured at 37°C in RPMI-1640 medium (Gibco^{TM}, USA) supplemented with 10% fetal calf serum (Gibco^{TM}, USA) and 1% Penicillin/Streptomycin (Gibco^{TM}, USA). In parallel experiments the cells underwent either irradiation (RT, 200 KV x-ray-irradiation, MG 420 Philips) or chemo-treatment (CT) with different concentrations of TMZ in the cultivation medium, or combined chemo/radiation-treatment (CT/RT). A scheme of these experiments is presented in Table 1. After each treatment surviving cells were harvested, washed with PBS, counted, aliquoted and stored at −80°C until protein analysis.

Western-blot analysis

Untreated (control) and treated glioma cells in non-confluent (day 4 of growth) and confluent (day 9) cultures were lysed by homogenisation in lysis buffer (9 M urea, Merck, Germany), 1% DTT (Sigma, USA), 2% CHAPS (Merck, Germany), 0.8% Bio-Lyte, pH 3–10 (Bio-Rad, USA), 5 mM Pefabloc (Merck, Germany) followed by a centrifugation step. The protein concentration was quantified by the DC-Protein Assay (Bio-Rad, USA). Forty μg protein of each sample were loaded onto 12% SDS-polyacrylamide gels and electrophoresed to separate proteins. The proteins were then transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, UK) and afterwards incubated at room temperature in blocking-buffer (58 mM $NaHPO_4$, 17 mM NaH_2PO_4,

Table 1. Scheme of cell culture experiments: all experiments were repeated 6-times. Control means untreated glioma cells; RT – irradiation; CT – chemo-treatment with TMZ; CT/RT – combined treatment performed as chemo-treatment with either 10 μg/ml TMZ (CT10/RT) or 30 μg/ml g/ml TMZ (CT30/RT) followed by irradiation; RT/24 – 24 hours is the incubation time after irradiation

Name of untreated cells	Time of cultivation before the treatments, days	Concentration of TMZ in medium, μg/ml	Time of chemo-treatment before the analysis, h	Incubation time with TMZ before irradiation, h	Number of irradiation applications each with 2 Gy	Incubation time after irradiation before the analysis, h
Control 4	4	0	0	–	0	0
Control 9	9	0	0	–	0	0
RT/2	4	0	0	–	1	2
RT/2	9	0	0	–	1	2
CT10/2	4	10	3	–	0	0
CT30/2	4	30	3	–	0	0
CT10/2	9	10	3	–	0	0
CT30/2	9	30	3	–	0	0
CT10/RT/2	4	10	3	1	1	2
CT30/RT/2	4	30	3	1	1	2
CT10/RT/2	9	10	3	1	1	2
CT30/RT/2	9	30	3	1	1	2
CT10/RT/24	4	10	24	1	1	23
CT30/RT/24	4	30	24	1	1	23

68 mM NaCl, 5% nonfat dry milk powder; 0.1% Tween 20) for 1 hour. Primary antibody incubation was performed at room temperature using a 1:250 dilution of anti-human-ABC-1 transporter (Santa Cruz, USA) in washing buffer I (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 1% non-fat dry milk powder, 0.1% Tween 20) for 1 hour. The membranes were then washed four times in the same solution. The horseradish peroxidase-labelled anti-goat secondary antibody was incubated at room temperature with the membranes in washing buffer I followed by three washes in washing buffer II (58 mM $NaHPO_4$, 17 mM NaH_2PO_4, 68 mM NaCl, 1% nonfat dry milk powder; 0.3% Tween 20) and three washes in washing buffer I. Then the membranes were reacted with chemiluminescent reagent ECL plus (Detection Kit, Amersham Biosciences, UK) for 1 hour and processed for auto-radiography. The individual signals were measured densitometrically using "Quantity One" imaging system (Bio-Rad, USA).

Results

Cellular density of untreated and treated U87-MG cells

The growth curves are shown in Fig. 1. The density of untreated cells were increasing exponentially until plateau was reached at day 9 of growth (confluent cultures). Similar exponential growth was observed also for irradiated cells being, however, about 1.1 to 2-times slower in exponential phase and 1.3-times slower in plateau phase compared to untreated cells. In contrast the density increase of chemo-treated cells as well as that of the cells, which underwent the combined CT/RT treatment was of linear nature. From all four linear curves the highest cellular density was observed under simple chemo-treatment with low TMZ concentration (CT10) and middle density under either simple chemo-treatment with high TMZ concentration (CT30) or under combined treatment with low TMZ concentration (CT10/RT). The lowest cellular density was shown under combined TMZ treatment with high TMZ concentration (CT30/RT) being about 1.4-, 2-, 2.4-, 7-, and 9-times lower compared to that of CT10/RT, CT30, CT10, RT, and untreated cells in confluent cultures respectively. The middle of exponential growth of untreated cells was observed at day 4 of growth, where the cellular density was 1.8-times lower compared with that in plateau phase. Cells at day 4 of growth were further taken for protein investigations as non-confluent cultures and compared with confluent cultures in plateau phase at day 9 of growth.

Relative expression levels of ABC-1 transporter

The relative expression levels of ABC-1 are shown in Fig. 2. Protein quantification analysis revealed a considerably increased ABC-1 expression by both growing cell density and cell treatments applied. Untreated cell cultures (named "controls" in Fig. 2) expressed approximately 13-times less target protein at day 4 of growth (non-confluent cultures) than at day 9 (confluent cultures). The increase in the target expression after application of cell treatment was about 1.25 to 20 times dependent on the growth phase (non-confluent/confluent cultures) and kind of treatment. After cell treatments, the production of the protein was only 1.25- to 2-fold enhanced in confluent

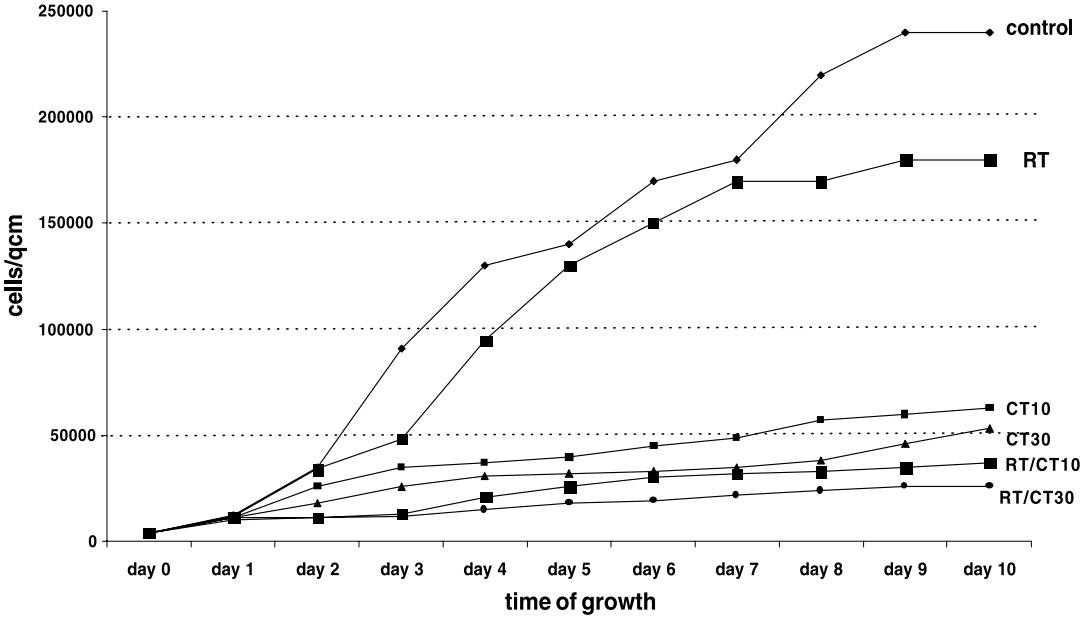

Fig. 1. Glioma cell growth/density curves. Names and performance of different cell treatments are explained in Table 1

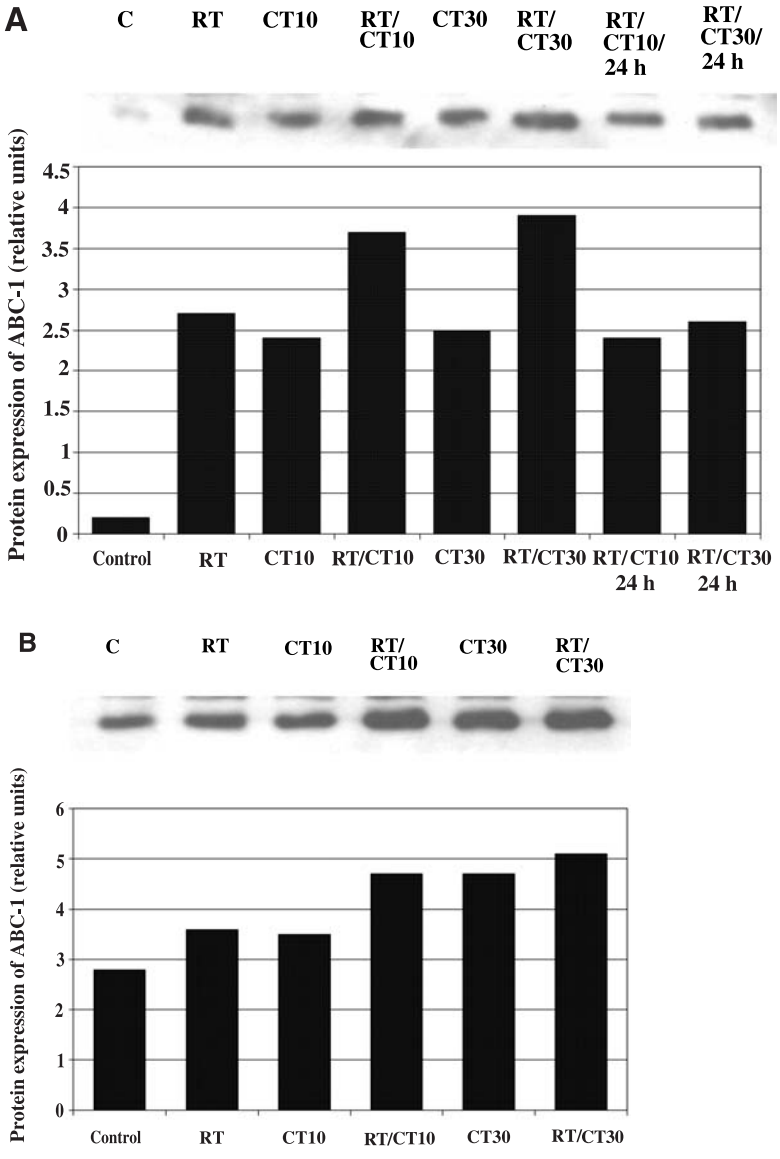

Fig. 2. Western-blot protein expression analysis of ABC-1 in untreated (*C*) and treated (see Table 1) glioma cells with corresponding evaluation for **A** non-confluent cultures at day 4 of growth, and **B** confluent cultures at day 9 of growth

cultures, whereas in non-confluent cultures it was 12- to 20-times higher in treated compared to untreated cells. The lowest expression levels were observed in untreated non-confluent cultures. In contrast, the highest levels were monitored in confluent cultures after irradiation followed by application of the highest drug concentration (30 μg/ml of TMZ in CT30/RT). Only minor expression differences were shown among confluent cultures treated simply with the highest drug concentration or alternatively with combined therapy (either CT30/RT or CT10/RT) being independent on the drug concentration applied. These expression levels were about 1.5-times higher than those in confluent cultures which were either irradiated or treated with 10 μg/ml of TMZ. Similarly in non-confluent cultures, the protein levels measured in cells treated simply with RT or CT were approximately 1.5-times lower than those of cells which underwent the combined CT/RT treatment. Interestingly under simple chemo-treatment the response of the ABC-1 expression to TMZ supplementation was strongly dependent on the applied drug concentration only in confluent cultures but not in non-confluent cultures. Both CT10 and CT30 increased the ABC-1 protein levels up to 12.5-times compared to those of untreated cells in non-confluent cultures. The inducible effect of the combined treatment decreased 1.5-fold within 24 hours after application, demonstrating, however, still approximately 13-times higher expression levels compared to the baseline.

Discussion

Among tumour patients who received chemotherapy, positive expression of ABC transporters was shown to correlate significantly with poor outcome (Chou et al., 1995; Yamaguchi et al., 1995; Bera et al., 2002). In this work we investigated an effect of both irradiation (RT) and chemo-treatment (CT) as well as combined chemo/radiation treatment (CT/RT) on the expression of the ABC-1 transporter in human U87-MG glioma cells. The ABC-1 transporter may play an important role in both hydrophobic drug export and regulation of endothelial function in blood-brain barrier. Whereas secondary glioblastomas show frequent p53 gene mutations, *de novo* (primary) glioma tumours are usually independent of p53 alterations (Horiguchi et al., 2001). Therefore, the p53-wild-type U87-MG human glioma cell line has been chosen for our investigations. For the chemo-treatment of U87-MG cells temozolomide (TMZ), a new alkylating agent with improved anti-tumour activity, was used. The cytotoxicity of TMZ appears to be mediated mainly through the creation of O^6-meG in genomic DNA (Denny et al., 1994; Wedge et al., 1996). This alkylating agent has been recently introduced into Phase II and III trials for treatment of recurrent high-grade gliomas and has been shown to yield objective response or stable disease in 50–60% of gliomas (Bower et al., 1997; Paulsen et al., 1999; Yung et al., 1999; Osoba et al., 2000). However, the development of effective TMZ-based chemo-therapeutic regiments for gliomas is limited by a relative lack of understanding of the action of TMZ in glioma cells on the one hand and the mechanisms of TMZ-resistance in these cells on the other hand.

Our results show that TMZ treatment up-regulates significantly the level of ABC-1 expression in malignant glioma cells. Moreover, also irradiation and even the cellular density demonstrated activating effects on the level of ABC-1 expression in these cells.

Controlled regulation of multidrug-resistance proteins is an important component of successful anti-tumour approaches. ABC-1 is a member of the group of transporters, which are known to be involved in the export of anti-cancer drugs and, therefore contribute to the resistance of cancer cells towards chemo-therapy. These proteins are highly homologous with similar structures of traffic ATPases, which use ATP to drive the transport of a wide variety of substances across the membrane, including amino acids, peptides, metals, phospholipids, toxins, antibiotics, etc. The functional unit contains two distinct transmembrane regions and two distinct nucleotide-binding domains (Mengerink and Vacquier, 2002). Promotor activity increases in response to various environmental stimuli and stress factors in a manner that is dependent on the inverted CCAAT box (Kohno, 1997).

Our results show that TMZ treatment, irradiation and even the cellular density act as stress factors considerably up-regulating the level of ABC-1 expression in malignant glioma cells. This up-regulation obviously involves specific nuclear factors interacting with the promotor region under stress conditions. The stress-dependent induction was shown by the promoter activity of a homologous ABC transporter, the 170-kDa plasma membrane protein MDR-1 (Kohno, 1997). The binding activity of the nuclear factor MDR-NF1 that interacts with the promotor region of MDR-1 was demonstrated in nuclear extracts of human cells treated with either anti-cancer drugs or UV-light (Kohno, 1997). The amino acid sequence encoded by the cloned cDNA for MDR-NF1 was identical to that of the human Y-Box binding protein 1 which is sensitive to cross-linking DNA damage.

However, in spite of high structural and functional similarities among the members of the ABC transporter family, a differential tissue and signal specific expression of ABC transporters has been observed. Thus, elevated expression of the membrane transporter p-glycoprotein (Pgp) and impaired expression of the nuclear enzyme topoisomerase II (topo II) are well-known mechanisms for *in vitro* acquired drug resistance. However, Pgp levels determined by Western blot analysis in leukaemic cells showed no correlation with induced DNA damage (Zhou et al., 1999). Screening for tissue specific ABC transporters, which potentially may decrease the sensitivity of target tumour cells towards an applied treatment, is an essential part in the improvement of each anti-cancer approach. The present work is the first report on up-regulation of the ABC-1 transporter in human malignant glioma cells in response to DNA damaging agents in a dose dependent manner. Its expression, therefore, may considerably decrease the irradiation and TMZ sensitivity of these cells under the applied therapy. Our data contribute to the explanation of the cross-resistance of glioma cells towards drugs and irradiation observed by other authors (Denecke et al., 1997) and make the ABC-1 transporter an attractive pharmacological target for a clinical breakthrough in the therapy of malignant gliomas.

It has been shown that glioma patients with an increased rate of malignant cell proliferation generally have a worse prognosis, whereas a low cell density showed trends with regard to being associated with longer survival (Prayson et al., 2000). However, the functional

link between the higher cell density and rapid disease progression has not been clarified yet. In this work we demonstrate that in non-confluent cell cultures the up-regulation of ABC-1 expression in response to TMZ treatment is almost independent of the applied TMZ-concentration. In contrast, in confluent cultures this response correlates well with the applied concentration of the drug. This finding provides the first clear evidence for a link between quorum sensing and multidrug efflux for malignant glioma cells similar to prokaryotic species, where drug efflux pumps mediate cell-cell communication in response to cell density (Rahmati et al., 2002). This observation is important particularly for therapeutic approaches of primary gliomas since the U87-MG cell line, like the majority of primary gliomas, has p53-wild-type phenotype, is able to accumulate the functional p53 in response to stress, and is more resistant towards therapeutic chemo/radiation-treatment than p53-mutant gliomas (Kono et al., 2002). Taken together, we would expect that an application of specific antagonists of the drug efflux pump conducted by ABC-1 in gliomas might simultaneously reverse the multidrug resistance, up-regulate irradiation sensitivity and down-regulate the density of the malignant cellular community.

Acknowledgements

The authors thank Mrs. K. Kim for her assistance in the laboratory work.

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