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Cystathionine Promotes the Proliferation of Human Astrocytoma U373 Cells

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Abstract. *Background/Aim: In certain cancers, accumulation of cystathionine has been observed. The present study investigated the effect of cystathionine on astrocytoma (U373) cell proliferation, the activity of γ-cystathionase (CTH) and changes in thiols levels. Materials and Methods: The methods used in the study included cytotoxicity assay, crystal violet staining method, CTH activity assay and reverse phase-high performance liquid chromatography (RP-HPLC). Results: The addition of cystathionine to the culture medium resulted in an increase of cystathionine level in U373 cells after 24 h of culture. Reduction of intracellular cystathionine level after 48 and 72 h of culture was associated with increased L-cysteine and L-cystine levels and stimulation of cell proliferation. Interestingly, a decrease in intracellular L-cysteine and L-cystine levels during the first hours of culture was observed. Conclusion: Elevated levels of cystathionine resulted in increased U373 cell proliferation by increasing the L-cysteine levels and GSH/GSSG ratio (especially after 72 h of the culture), but not with a simultaneous increase in the levels of total glutathione.*

Cystathionine is known as an intermediary metabolite in L-cysteine synthesis from methionine through the transsulfuration pathway. Endogenous synthesis of cystathionine, a sulfur-containing metabolite, is catalyzed by cystathionine β-synthase (CBS) *via* β-replacement reaction in which serine condenses with homocysteine in a pyridoxal-5'phosphate–dependent manner. Cystathionine is subsequently converted to L-cysteine, α-ketobutyrate and ammonia by the action of cystathionine γ-lyase (CTH) (Figure 1). L-cysteine is catabolized by desulfuration pathways (generating sulfane sulfur-containing compounds and H_2S) and oxidative pathways (production of taurine and sulfate) and is used for glutathione and proteins synthesis. Under physiological conditions, L-cysteine can substitute for serine, and the β-replacement of the sulfhydryl group of L-cysteine with homocysteine also results in cystathionine formation with release of H_2S (Figure 1) (1-3).

Cystathionine exerts anti-inflammatory (4) and anti-apoptotic effects (5-8), is involved in eliminating superoxide radicals (9-11) and plays a role in liver protection against endoplasmic reticulum stress-induced injury (6, 12).

Cystathionine is a recently discovered substrate of cystine/glutamate transporter (system xc^-) and may play an important role in regulating extracellular glutamate homeostasis in the brain (13). It is uncertain whether cystathionine serves as a direct precursor of L-cysteine for glutathione or whether it may replace incoming L-cystine at the level of the xc^- exchanger. It is also uncertain whether cytosolic cystathionine itself is derived primarily from methionine by the transsulfuration pathway or if it enters the cells in place of L-cystine *via* the exchanger (14). The xc^- system plays significant roles in the growth and proliferation of cancer cells (15).

Massive accumulation of cystathionine and subsequent cystathioninuria have been observed as frequent and highly specific markers of neuroblastoma (16-19). Sen *et al.* (5) reported that cystathionine was selectively enriched in the breast cancer tissues as compared with undetectable levels in the normal breast tissues (5). Higher serum cystathionine as well as homocysteine and L-cysteine concentrations are also risk factors for metastatic prostate cancer progression and could serve as pre-surgical markers for aggressive disease (20). Wróbel *et al.* (21) reported that an average cystathionine level in higher grade human gliomas (II/III, III/IV and IV) was higher in comparison to low grade gliomas (II).

The purpose of the research undertaken in this study was to clarify whether the increase in cystathionine levels in cancer cells would affect their proliferation.

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Key Words: Cystathionine, brain cancer cells, thiols, sulfurtransferases, hydrogen sulfide.

Materials and Methods

Sources of chemicals. D,L-Cystathionine, bathophenanthroline-disulfonic acid (BPDS), 2,4-dinitrofluorobenzene (DNFB), acetonitrile, reduced nicotinamide adenine dinucleotide (NADH), lactate

dehydrogenase (LDH), pyridoxal phosphate (PLP), Folin–Ciocialteau reagent, trypsin, Dulbecco's Modified Eagle Medium (DMEM), and crystal violet (N-hexamethylpararosaniline) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Fetal bovine serum and penicillin/streptomycin were obtained from GIBCO Laboratories (Grand Island, NY, USA). Trifluoroacetic acid (TFA) and 2-mercaptoethanol were obtained from Flucka Chemie GmbH. Ne-methyl-L-lysine was purchased from Bachem (Bubendorf, Switzerland). All other chemicals were of reagent grade and purchased from common commercial suppliers.

Cell culture. Human astrocytoma U373 cells (ECACC, UK) were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin (100 Units/ml penicillin and 100 μg/ml streptomycin), at 37˚C in humidified 95% air and 5% CO_2.

Cell homogenization. For determination of the level of reduced (GSH) and oxidized (GSSG) glutathione, L-cysteine, L-cystine and cystathionine, U373 cells were suspended in 0.1 ml 10% perchloric acid/1 mM BPDS. The sediment was separated by centrifugation at 1600 g for 10 min, and the supernatant was saved at –80˚C until used for *Reverse Phase-High Performance Liquid Chromatography* (RP-HPLC) analyses.

For determination of CTH activity and protein level, U373 cells were suspended in 0.1 M phosphate buffer pH 7.5, in the ratio of 1×10^6 cells/ 0.04 ml of the buffer, and sonicated 3×5 sec at 4˚C (Bandelin Sonoplus GM 70). After centrifugation at 2800 g at 4˚C for 10 min, the supernatant was used for analysis.

Determination of cell viability. The effect of cystathionine on cell viability was assessed by measuring the leakage of lactate dehydrogenase (LDH) from dead or dying cells using a Cytotoxicity Detection Kit (Roche Applied Science, Penzberg, Germany) as described previously (22). The 0.25, 0.5 and 1 mM concentrations of cystathionine that yielded LDH leakage of less than 5% were used for the experiments.

Cell proliferation. The U373 cells were seeded on 96-well plates at a concentration of 2×10^3 cells/well in DMEM supplemented as reported above. Following 24 h incubation, the culture medium was replaced with 100 μl of culture medium (as a control) or 100 μl of medium containing 0.25, 0.5 or 1 mM cystathionine and the plates were cultured for 24, 48 and 72 h. Cell proliferation was examined using the modified crystal violet staining method (23). The absorbance was measured using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

CTH assay. CTH activity was determined according to Matsuo and Greenberg (24) as modified by Czubak *et al*. (25). The enzyme activity is expressed as nmoles of α-ketobutyrate formed during 1 min incubation at 37˚C per 1 mg of protein. Protein concentration in the homogenates was determined by Lowry (26) method.

RP-HPLC analysis. The levels of non-protein thiols (L-cysteine, L-cystine, GSH and GSSG) and cystathionine were determined by the method of Dominick *et al*. (27), with some modification as described by Bronowicka-Adamska *et al*. (28).

Statistical analysis. All the experiments were repeated at least three times. Data are expressed as the means±standard deviation (SD).

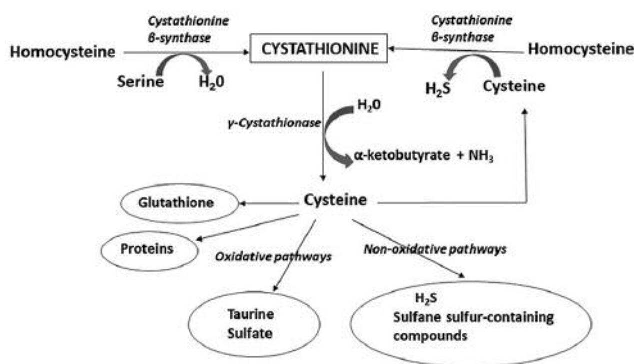

Figure 1. *Metabolic pathways involved in cystathionine synthesis and catabolism*.

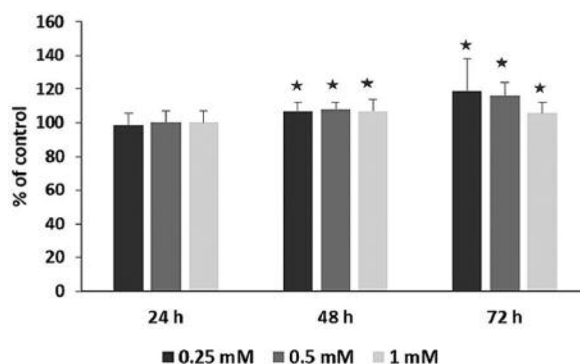

Figure 2. *The effect of cystathionine on the proliferation of the U373 cells. The U373 cells were incubated for 24, 48 and 72 h at various concentrations (0.25, 0.5, 1 mM) of cystathionine. Cell proliferation was analyzed by the crystal violet assay. The data presented in the Figure are the mean±SD of four independent experiments. *p<0.05 (Student's t-test) was considered statistically significant as compared with the non-treated controls.*

Statistical analyses were performed using the Student's *t*-test. Differences were considered significant at $*p<0.05$.

Results

Effect of cystathionine on the proliferation of human astrocytoma U373 cell line. The U373 cells were cultured in the presence of various concentrations of cystathionine within 24, 48 and 72 h, and subsequently, the crystal violet method was used for studying the cell proliferation. As shown in Figure 2, the proliferation of cystathionine-treated cells increased after 48 and 72 h of the culture. In the presence of 0.25, 0.5, and 1 mM cystathionine, the U373 cell proliferation was increased respectively about 7%, 6% and 7% (after 48 h of the culture), and about 19%, 16%, and 6% (after 72 h of the culture). Cystathionine did not affect the proliferation of U373 cells after 24 h treatment.

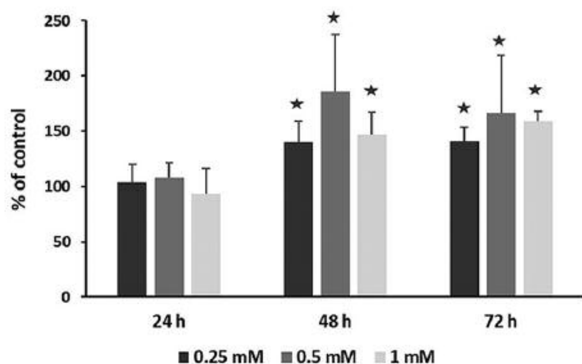

Figure 3. *Cystathionine effect on γ-cystathionase activity in the U373 cells. The cells were treated with various concentrations of cystathionine (0.25, 0.5, 1 mM) for 24, 48 and 72 h. Every value represents the mean±SD of four independent experiments; *p<0.05 (Student's t-test). In the U373 cells, γ-cystathionase activity determined after 24, 48 and 72 h of the culture equaled, respectively, 398±52, 437±128, and 373±156 nmole x mg protein-1 x min-1 (control values).*

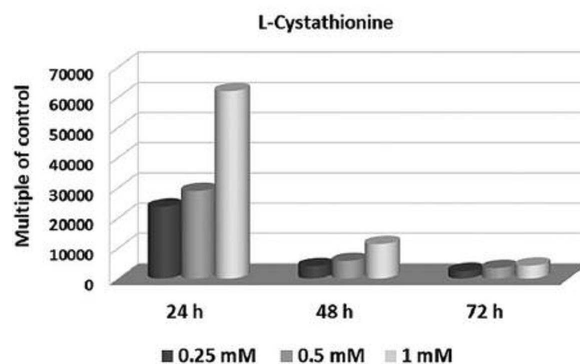

Figure 4. *Cystathionine effect on the intracellular level of cystathionine in the U373 cells. The cells were incubated for 24, 48 and 72 h in the presence of various concentrations of cystathionine (0.25, 0.5, 1 mM). Every value represents the mean±SD of three-four independent experiments. The intracellular level of cystathionine determined in the control U373 cells equaled to 0.41±0.21 nmole/10^6 cells.*

Effect of cystathionine on γ-cystathionase activity in the human astrocytoma U373 cell line. The results presented in Figure 3 show changes in γ-cystathionase activity in the U373 cells in the presence of cystathionine (0.25, 0.5, and 1 mM) during the culture. A significantly increased activity of CTH was observed in the U373 cells cultured for 48 and 72 h with cystathionine as compared to the control cells. For 0.25, 0.5, and 1 mM cystathionine, CTH activity was increased by about 40%, 86%, 47% after 48 h of cystathionine treatment, and by about 41%, 66%, 59% after 72 h, respectively.

Effect of cystathionine on the intracellular level of cystathionine, L-cysteine, L-cystine, GSH/GSSG ratio and total glutathione in the human astrocytoma U373 cell line. The RP-HPLC method was used for the determination of cystathionine, L-cysteine, and L-cystine levels in the U373 cells. As compared to the cells cultured in DMEM without cystathionine, the addition of cystathionine (0.25 mM, 0.5 mM, 1 mM) resulted in the increase of the intracellular cystathionine levels in a concentration-dependent manner (Figure 4). The levels of cystathionine detected after 24 h were higher than after 48 h and 72 h. As illustrated in Figure 5, during the first 24 h of the culture, L-cysteine and L-cystine levels in the U373 cells were significantly decreased in the presence of 0.5 mM and 1 mM cystathionine compared to the untreated cells (reduction of L-cysteine about 10-65%; reduction of L-cystine about 15-40%). However, in the U373 cells treated with 0.5 mM and 1 mM cystathionine for 48 h and 72 h, there was a time-dependent decrease in intracellular cystathionine levels (Figure 4), associated with increased L-cysteine and L-cystine levels (Figure 5). The GSH/GSSG ratio was increased in the presence of cystathionine in a time-dependent manner

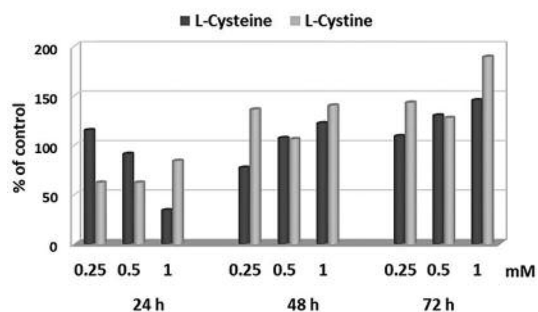

Figure 5. *Cystathionine effect on the intracellular level of L-cysteine and L-cystine in the U373 cells. The cells were incubated for 24, 48 and 72 h in the presence of various concentrations of cystathionine (0.25 mM, 0.5 mM, 1 mM). Every value represents the mean±SD of three-four independent experiments. The intracellular level of L-cysteine and L-cystine determined in the control U373 cells equaled, respectively, 0.32±0.04, and 2.51±0.96 nmole/10^6 cells.*

(Figure 6A), however the level of total glutathione was unchanged (Figure 6B).

Discussion

The results of this study demonstrated that cystathionine promotes the proliferation of the human astrocytoma U373 cells in a time dependent-manner (Figure 2) and was associated with increased intracellular L-cysteine and L-cysteine levels (Figure 5) and GSH/GSSG ratio (Figure 6A). Interestingly, lack of simultaneous increase in the total glutathione level in the cystathionine-treated U373 cells (Figure 6B) suggests that cystathionine as a substrate of

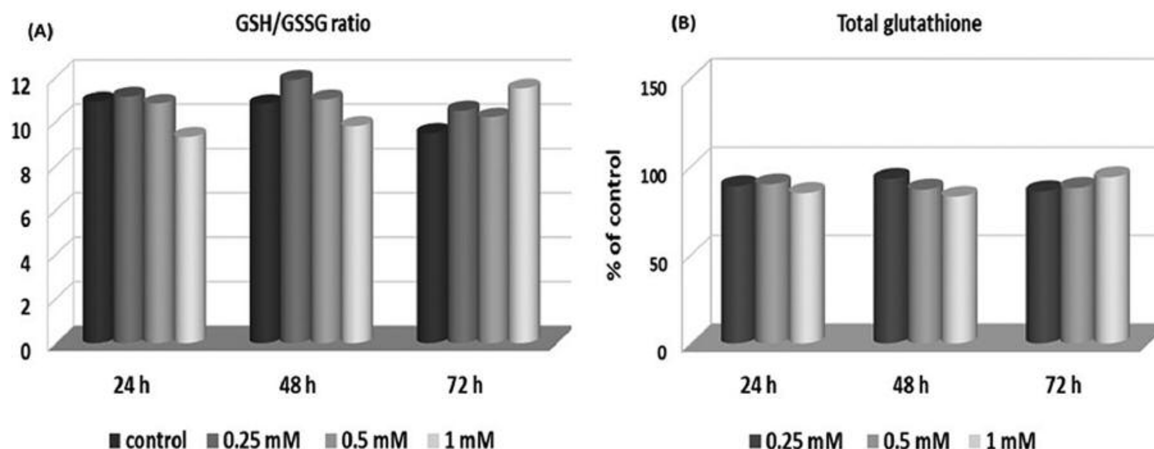

Figure 6. *Cystathionine effect on the GSH/GSSG ratio (A) and the total glutathione (B) in U373 cells. The cells were incubated for 24, 48 and 72 h in the presence of various concentrations of cystathionine (0.25, 0.5, 1 mM). Every value represents the mean±SD of three-four independent experiments. The intracellular level of GSH and GSSG levels determined in the control U373 cells equaled, respectively, 12.45±1.71, and 1.51±0.34 nmole/10^6 cells.*

cystine/glutamate transporter (13) can compete with cystine for this transporter, also affecting the level of glutamate and consequently limiting the synthesis of glutathione. It was shown (29) that L-cysteine has a potential to stimulate proliferation and increase differentiation of the neural stem cells to neurons and astroglia. L-cysteine-induced proliferation was associated with the phosphorylation of extracellular signal-regulated kinases 1/2 (ERK 1/2) and with altered expression of differentiation-related genes. L-cysteine can enhance proliferation and differentiation of the neural stem cells *via* the cystathionine β-synthase/H_2S pathway (29). H_2S can also be generated from L-cysteine and L-cystine *via* various γ-cystathionase catalyzed reactions (30). Exogenous L-cysteine and L-cystine also promoted proliferation of the human colon cancer cells (31). Other reports showed that exogenous cystathionine protected macrophages against apoptosis induced by oxidized low-density lipoprotein (ox-LDL) (7) and can inhibit apoptosis induced by tunicamycin (the endoplasmic reticulum stress inducing agent) in the human embryonic kidney cells (6). Sen *et al*. (5) reported that cystathionine protected the human breast cancer cells against excess reactive oxygen species and doxorubicin-induced apoptosis. Cystathionine inhibited mitochondria-mediated apoptosis *via* increasing mitochondrial membrane potential, inhibiting MPTP (Mitochondrial permeability transition pore complex) opening, suppressing cytochrome c release from the mitochondria into the cytoplasm, as well as downregulating caspase-9 activities and caspase-3 protein expression (5, 7).

It was also observed that the addition of cystathionine to the culture medium resulted in an increase in the intracellular cystathionine levels in a concentration-dependent manner (Figure 4), which confirmed its transport through the cell membrane. The highest level of cystathionine in the U373 cells was detected after 24 h of culture and then it gradually decreased after 48 and 72 h (Figure 4). Our previous results showed (32) that the activity of γ-cystathionase (CTH) in the U373 cells was low - about 0.4 nmole × mg protein^{-1} × min^{-1}. Addition of cystathionine resulted in an increase in the CTH activity after 48 and 72 h of treatment (Figure 3), and as a consequence, the level of L-cysteine in the cells was also elevated after 48 and 72 h (Figure 5). Interestingly, during the first 24 h of treatment, a high level of cystathionine in the U373 cells (Figure 4) was associated with the reduction of the intracellular L-cysteine and L-cystine levels (Figure 5). This suggests that cystathionine inhibits L-cysteine uptake in the U373 cells at the beginning of the culture when the cystathionine concentration in the medium is the highest. Kobayashi *et al*. (13) reported that in embryonic fibroblasts derived from wild-type embryos, L-cysteine uptake was significantly inhibited by cystathionine in a concentration-dependent manner. Kim *et al*. (33) showed that L-cystine uptake by the TcyABC transporter was also strongly inhibited by cystathionine in *Streptococcus mutans*.

In view of the fact that cystathionine promotes proliferation of U373 cells, the idea of using selective cystathionine β-synthase inhibitors, such as the natural biflavonoid compound sikokianin C, a recently described inhibitor (34), for inhibiting the proliferation of human brain cancer cells seems promising.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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