IN VITRO EFFECTS OF SELENIUM ON HUMAN GLIOBLASTOMA MULTIFORME CELL LINES: A PRELIMINARY STUDY

Duygu Harmanci¹,³, Zubeyde Erbayraktar², Oya Sayin³ and Gul Akdogan Guner⁴

¹Dokuz Eylul University Graduate School of Health Sciences, Department of Molecular Medicine,
²Dokuz Eylul University School of Medicine, Department of Medical Biochemistry,
³Dokuz Eylul University Research Laboratory R-LAB;
⁴İzmir University of Economics, School of Medicine, Izmir, Turkey

SUMMARY – Glioblastoma multiforme (GBM) is caused by the central nervous system-derived glial cells, and represents the most common (50%-60%) form of primary brain tumors. The aim of this study was to investigate the in vitro effects of selenium on human GBM cells. In the present study, GMS-10 and DBTRG-05MG human GBM cell lines were used as a model to examine selenium entering the cell, cell proliferation, cytotoxicity, DNA fragmentation and Ki-67 protein expression in selenomethionine treated and non-treated groups. Seleno-L-methionine (SeMet) as the organic source of selenium exerted effects on cell proliferation and cytotoxicity, as assessed with WST-1 and lactate dehydrogenase (LDH) tests, respectively. Apoptosis was assessed by DNA fragmentation with an enzyme-linked immunosorbent assay. Ki-67 protein expression was determined by Western blotting, while selenium measurements were performed in the supernatants and lysates by using Graphite Furnace Atomic Absorption Spectrometry. This is the first study to examine the effects of SeMet on cell proliferation and death in GMS-10 and DBTRG-05MG cells. Both GBM cell lines responded to SeMet in a dose- and time-dependent manner. WST-1 test showed that low-dose SeMet treatment (50 and 100 μM) increased cell proliferation. Analysis of intracellular SeMet levels by using AAS showed results consistent with viability and cytotoxicity tests. SeMet treatment for 72 h caused increased DNA fragmentation in both cell lines. In conclusion, our results suggest that SeMet induces cell death at high doses, while increasing cell proliferation at low doses. In the view of the data obtained in this investigation, further studies focusing on the possibility of using SeMet against different types of GBM and in combination with prospect synergic compounds are considered to be worthwhile.

Key words: Glioblastoma – therapy; Anticarcinogenic agents; Humans; Selenium; Selenomethionine; Cell proliferation – drug effects; Cell death – drug effects; Cytotoxicity; Ki-67 antigen

Introduction

Selenium is an essential trace element and an extraordinary element¹,². Its own codon enables its incorporation into proteins as selenocysteine³. Selenium functions in antioxidant mechanisms, immune systems, detoxification mechanisms, inflammation, male fertility, and also exerts anti-cancer effects⁴. Several studies have shown that certain selenium compounds, such as selenite and seleno-L-methionine, have roles as chemopreventive agents in different cancers, including prostate, melanoma, lung, esophageal and gastric-cardia cancers⁵,⁷.

Glioblastoma multiforme (GBM) is the most aggressive brain cancer due to its capacity of rapid inva-
sion and its strong resistance to therapy. Median survival is 10 to 12 months. Some patients can live up to 14 months. Several reports have shown that treatment with antioxidants is beneficial in preventing tumor growth. Recently, temozolomide, bevacizumab, and other alkylating agents are used in the treatment of glioblastoma, but drug resistance is the most important problem. To overcome this therapeutic challenge, new approaches involving a combination of anti-cancer drug and antioxidant treatment have been developed.

Previous studies have investigated, both in vitro and in vivo, the preventive and therapeutic effects of selenium on different cancers, including prostate, lung, colon, breast, melanoma, and various brain tumors. Inorganic forms of selenium, including sodium selenite and sodium selenate, have been used in these studies.

To our knowledge, seleno-L-methionine (SeMet), the organic form of selenium, has not been tested in any study. We hypothesized that SeMet would be effective in controlling cell proliferation and apoptotic cell death. Here, we investigated, on two different human GBM cell lines, the effects of SeMet on proliferation, cytotoxicity, and Ki-67 protein expression. In addition, correlation with the amount of selenium entering the cell (following treatment with different doses of SeMet) was also evaluated.

Materials and methods

Test chemicals

Seleno-L-methionine (MW 196.11 g/mol, CAS Number 3211-76-5; Sigma, USA) was dissolved in distilled water. Then, the solution was filtered and diluted as required. All dilutions were carried out using the medium. All treatments were carried out at final concentrations of 50, 100, 500 and 1000 μM.

Cell culture

Adherent human GBM cells, GMS-10 and DBTRG-05MG, were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). GMS-10 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, D6046; Sigma; USA), while DBTRG-05MG cells were cultured in RPMI 1640 (HyClone, Thermo Scientific; USA) supplemented with 10% fetal calf serum (Biological Industries, Israel), 1% L-glutamine (G7513; Sigma; USA), and 1% penicillin-streptomycin (P4333; Sigma; USA) at 37 °C in 95% air and 5% CO2 incubator. Cells were trypsinized at a split ratio of 1:2 or 1:3.

SeMet treatment

Both cell lines were treated with different concentrations of SeMet (50-1000 μM) for 24, 48 and 72 h. Afterwards, cell survival was assessed qualitatively by light microscopy.

Cell viability assay

The inhibition of cell growth was determined by [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] kit (WST-1 05 015 944 001, Roche). All GBM cells were seeded on 96-well plates (1x10^4 cells/well) and treated with 50, 100, 500 and 1000 μM SeMet for 24, 48 and 72 h. Then, 10 μL of WST-1 stock solution was added to each well. Cells were incubated at 37 °C for 0.5-4 h. After incubation, cell viability was assayed by measuring the absorbance at 440 nm on a microplate reader (Bio-Tek Instruments, Inc., USA). The results were expressed as mean optical density (OD) of experimental groups (n=5) vs. control (non-treated cells). The mean OD of the control group was set to represent 100% cell viability.

Cell death assay

Cell death was assayed by using lactate dehydrogenase (LDH) assay (LDH 11 644 793 001, Roche). Quantifying the amount of LDH in the medium from dead cells and dividing the result by the total amount of LDH in the medium and cell lysate in the sample calculated the cell cytotoxicity percentage. Cell cytotoxicity results were expressed as the percentage of LDH released. All GBM cells were seeded on 96-well plates (1x10^4 cells/well) and treated with 50, 100, 500 and 1000 μM SeMet for 24, 48 and 72 h. Then, a 10-μL lysis solution was added to the wells to achieve maximal release in maximal group cells. Cells were incubated at 37 °C for 2 h for lysis. After incubation, LDH working solution was added to each well (supernatants without cell) and LDH released from the medium was detected by measuring absorbance at 490 nm using a microplate reader (Bio-Tek Instruments, Inc., USA).
**Cell death ELISA**

Apoptosis was assessed by DNA fragmentation with an enzyme-linked immunosorbent assay (Cell Death ELISA Plus Kit 11774425001, Roche). Briefly, 1x10^4 cells/well were seeded on 96-well plates and treated with 50, 100, 500, and 1000 μM SeMet for 24, 48 and 72 h. The plate was centrifuged at 600 g at 4 °C for 10 minutes and then the supernatants were removed. The cell pellet containing apoptotic bodies was lysed with 0.2 mL of a lysis buffer (cell death ELISA Plus, Roche) and incubated for 30 minutes at room temperature. Cell lysate solutions (20 μL) were placed in duplicate into the wells of the streptavidin-coated microplate, to which 80 μL of the immunoreagent containing a mixture of anti-histone-biotin and anti-DNA-POD was added. The plate was covered with an adhesive cover foil and incubated for 2 h at 25 °C in a shaking incubator at 300 rpm. During the incubation period, the anti–histone antibody binds to the histone component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated microplate via its biotinylation. At the same time, the anti-DNA-POD antibody reacts with the DNA component of the nucleosomes. The unbound antibodies were washed with the incubation buffer. The amount of nucleosomes retained by the POD in the immunocomplex, corresponding to the extent of apoptosis was quantitatively determined photometrically with ABTS (2,2’-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid) as substrate, using a microplate reader at a wavelength of 405 nm and reference wavelength of 490 nm. The enrichment of mono- and oligonucleosomes in the cytoplasm of the apoptotic cells is due to the fact that DNA degradation occurs several hours before the plasma membrane breakdown. Quantifying the average OD from dead cells and dividing the result by that of average control cells yielded the enrichment factor.

**Selenium measurement**

Cell lysates and supernatants, which were obtained from the cell lines after incubation, were collected for selenium measurements, which were done by using Graphite Furnace Atomic Absorption Spectrometry (GFAAS). After incubation, cell media were collected and transferred to sterile falcons and the cells were scraped with a cell scraper. The 1000-ppm selenium standard (Titrisol Merck) solution was diluted to 1 ppm in 0.1 M HCl and then prepared to buffer stock concentrations in the range of 25-800 μg/L. All these buffer stock solutions were diluted to working concentrations in the range of 5-200 μg/L with the matrix modifier which was composed of 190 mg palladium nitrate and 60 mg magnesium nitrate, 200 μL triton X-100 dissolved in 100 mL 0.1 M HCl. Before analysis, cell lysates were diluted (1:100) with the matrix modifier. The matrix modifier was used as blank. All standards and samples were then placed in the auto sampler vial for GFAAS analysis. The sample injection volume was 20 μL. Selenium was detected at 196 nm.

**Western blot**

Following SeMet incubation, the cell lines were lysed in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM MgCl₂, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 1 mM PMSF, aprotinin (1 μg/mL) and leupeptin (1 μg/mL)). After the cells had been lysed, the protein concentrations were measured using a BCA protein assay kit (Pierce BCA Protein Assay Kit 23225, Thermo Scientific). Equal amounts of total cellular protein samples (15 μg) were separated on a 6% SDS-polyacrylamide gel at 100 V and transferred onto a PVDF membrane for 2 h at 200 mA (Atto). The membranes were incubated with 5% non-fat dry milk and 3% BSA, specific primary antibodies at 1:250 and 1:500 directed to either Ki-67 (Santa Cruz, Cat No. sc-7844) and calnexin (Santa Cruz, Cat No. sc-6465) overnight at 4 °C, followed by incubation with appropriate HRP conjugated secondary antibodies (donkey anti-goat IgG-Horseradish Peroxidase Conjugated Affinity Purified Antibody, Santa Cruz, Cat No. sc-2020) at 1:1000 dilution in PBS for 1 h at room temperature and subsequent chemiluminescent detection using standard quality x-ray films.

**Statistical analysis**

All data were expressed as mean ± SD and corresponded to the results of at least three independent experiments. The average of five wells was taken for each parameter. The cells treated with the medium served as control (100% viable) and the remaining samples were expressed as viability percentage decrease.
or increase as compared with the control. Kolmogorov-Smirnov test was used to assess whether the variables fitted normal distribution and showed a non-normal distribution. One-way ANOVA was used to analyze differences among these non-normally distributed groups. Pearson’s correlation test was used to determine correlation among experimental parameters. P values <0.05 were considered as statistically significant. SPSS v.19.0 (Chicago, IL, USA) was used for statistical analysis.

Results

**SeMet treatment suppresses GBM cell proliferation in a dose- and time-dependent manner**

Two human GBM cell lines were treated with increasing concentrations of SeMet for 24, 48 and 72 h. Then, cell viability was assessed by the WST-1 assay. As shown in Figure 1A and B, SeMet stimulated proliferation of cell lines in a dose-dependent manner. For the GMS-10 cell line, low doses (50 μM and 100 μM) were found to induce proliferation at 24 h (Fig. 1A). We did not find significant difference between the SeMet treated two cell lines for 24 h, but a significant difference (p<0.05) was found for 48 h and 72 h (Fig. 1). After 72-h SeMet treatment, the IC₅₀ values were 500±200 μM for GMS-10 and 500±100 μM for DBTRG-05MG.

**SeMet treatment induces apoptosis in GBM cells**

The LDH assay showed that high dose SeMet treatment (500 μM and 1000 μM) induced cell death at 24 h. After 72 h, 50 μM SeMet resulted in 2.08±0.45% increase, 100 μM in 13.26±0.76% increase, 500 μM in 35.52±1.37% increase and 1000 μM in 50.10±2.29% increase in cell death for the DBTRG-05MG cell line compared with control. For the GMS-10 cell line, 50 μM SeMet resulted in no death, whereas 100 μM resulted in 3.99±0.65%, 500 μM in 10.0±0.89% and 1000 μM in 20.96±1.90% increase in cell death compared with control. Furthermore, we found negative correlation between the results of the LDH assay and WST-1 assay. The results of the LDH assay correlated negatively with the results of the WST-1 assay. Pearson’s correlation test results and the related r and p values are indicated in Table 1.

**DNA fragmentation: apoptotic effects of SeMet**

DNA fragmentation analysis showed that SeMet induced apoptosis in both GBM cell lines after 72-h incubation. To quantify and further support the finding that SeMet treatment causes cell death in GBM cells, we performed cell death detection by ELISA. Compared with controls, treatment of GMS-10 cells with SeMet at 500 μM and 1000 μM concentrations resulted in 2.94- and 4.03-fold increase in the induction of apoptosis, respectively.
(A) GMS-10 and (B) DBTRG-05MG cells were treated with increasing concentrations of SeMet, and cell death was assayed at different time points: (a) 24 h, (b) 48 h and (c) 72 h; quantification of apoptosis in (C) GMS-10 and (D) DBTRG-05MG cell lines induced by SeMet at different time points: (a) 24 h, (b) 48 h and (c) 72 h; quantifying the average optical density (OD) from dead cells and dividing the result by the average control cells yielded the enrichment factor. Data are presented as mean ± SD (n=5) (*p<0.05, **p<0.01 compared with control group).

Fig. 2. Seleno-L-methionine (SeMet) triggers apoptotic cell death in a dose-dependent manner.
Selenium uptake of the cells was assessed by measuring selenium levels in the supernatants (extracellular) and lysates (intracellular) by GFAAS. At each dose, SeMet uptake was highest at 48 h. However, there was no significant difference compared with the control group.

**Ki-67 protein expression**

The SDS-PAGE and Western blotting determined protein levels of Ki-67 following SeMet treatment. Ki-67 band images were shown in the GMS-10 cells at 24 h (UVP BioImaging System, UK).

**Discussion and Conclusions**

The ‘magic bullet theory’ proposed by Paul Ehrlich a century ago still remains valid in the treatment of cancer. According to Ehrlich, the magic bullet is a compound that targets a single certain oncoprotein in a highly specific fashion and does not damage healthy cells. Today, current research is focused on targeted molecular treatment alternatives. This situation is valid for GBM. The development of drug resistance is a common condition in GBM. The most important problem with drugs is associated with those that cannot pass the blood-brain barrier. Selenium can cross the blood-brain barrier and selenoprotein P is expressed in the brain. The chemical form of selenium...
is significant. Inorganic forms of selenium are more toxic than organic forms of selenium. Selenomethionine is an organic form of selenium possessing anticancer properties. First data came from the Nutritional Prevention of Cancer (NPC) study, which indicated that selenium compounds have chemopreventive properties, but then the SELECT study has reported debate on the issue34.

The GMS-10 glioblastoma cells used are cancerous brain cells derived from a GBM patient23. DBTRG-05MG cells were obtained from a GBM patient having undergone surgery after chemotherapy and radiotherapy24. GMS-10 cells are null for p53 gene, but DBTRG-05MG cells have not lost their heterozygosity for the p5323,24. After 24-h SeMet treatment, two tumor cells were affected differently. The 50 μM and 100 μM SeMet treatment of GMS-10 cells for 24 h unexpectedly exerted proliferative activity, but under these conditions DBTRG-05MG cells were not affected. Interesting enough, in a way contrary to the nature of antioxidants, SeMet did not fight the cancer cells at 50-μM and 100-μM doses. Similarly, Sayin et al. demonstrated that antioxidants accelerated lung cancer progression in mice35. In this study, this difference may have resulted from differences in p53 status.

In this investigation, SeMet treatment decreased cell proliferation and showed cytotoxicity in a dose- and incubation period-dependent manner in both cell lines (except for 50 μM and 100 μM SeMet treatment of GMS-10 cells for 24 h). Furthermore, these parameters correlated to a large extent with the level of SeMet having entered the cells.

Previous studies have shown that SeMet affected different cell types at different concentrations and incubation times. Redman et al.36 found that IC50 levels of 45 μM SeMet for MCF-7/S cells, 50 μM SeMet for UACC-375 cells, 130 μM SeMet for HT-29 cells, 40 μM SeMet for DU-145 cells, 65 μM SeMet for A549 cells and 1 mM SeMet for fibroblast cells inhibited 50% of the growth. Suzuki et al.37 applied SeMet in the range of 10-1000 μM to HSC-3, HSC-4, A549 and MCF-7. In the present study, the IC50 values for SeMet were 500±200 μM for GMS-10 and 500±100 μM for DBTRG-05MG. Given that GBM is the most aggressive and resistant primary brain tumor, such higher values are expected for GBM. The amount of selenium daily intake for normal people is 55 to 70 mg/day. The body can tolerate maximum selenium level of 400 mg/day38. The doses used in this study were slightly higher than the recommended daily allowance, but they were still within the tolerable range.

In reviewing the literature, Zhu et al.21 showed that selenite (0.01–100 μM) inhibited cell growth on C6

**Table 1. Pearson’s correlation analysis of cell death and cell proliferation after seleno-L-methionine (SeMet) treatment**

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<th>24 h</th>
<th>48 h</th>
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<td>r</td>
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<td>DBTRG-05MG</td>
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GMS-10 and DBTRG-05MG = human glioblastoma multiforme cell lines
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rat and A172 human glioblastoma cells for 24, 48 and 72 h. They also determined that A172 cells responded more sensitively than C6 cells. In another study, Zhu et al.\(^20\) showed that selenite induced apoptosis on A172 and T98 human glioblastoma cells. In this study, we found that after 24-h and 48-h SeMet treatment, DBTRG-05MG cell death increased significantly at high doses (500 and 1000 μM), but GMS-10 cells were not killed by SeMet after 24 h, whereas they were killed at the 500 μM dose significantly after 48-h incubation. After 72-h SeMet incubation, DBTRG-05MG cell death increased at 100, 500 and 1000 μM doses significantly, and GMS-10 cells were killed by SeMet on the same doses but not significantly. As a result of the WST-1 and LDH assays, negative correlation was found between the proliferation and cytotoxicity findings and these parameters correlated to a large extent with the level of SeMet entering the cells. The highest SeMet level in both cell lines was measured after 48-h treatment. These findings correspond to the results of proliferation assays and suggest that SeMet cytotoxicity appears to be explained in part by the induction of apoptosis. Interestingly, we observed a significant increase in apoptosis in both cell lines. This condition may be related to the cell p53 status and the development of SeMet resistance in GMS-10 cells. Seo et al.\(^39\) showed that SeMet could activate the p53 protein by a redox mechanism and SeMet differentially affected cells wild type or null for p53 genes. In the view of such information, our data must be supported by new experiments such as siRNA transfection and apoptosis assays.

Ki-67 is a well-known proliferation marker, such as cyclin E, cyclin D and proliferating cell nuclear antigen (PCNA)\(^36\). Previous studies evaluated Ki-67 protein levels by immunohistochemistry, ELISA or Western blotting\(^40\)-\(^42\). Jin et al.\(^43\) showed that Ki-67 was associated with cell growth and regulating proliferation in GBM. In the present study, Ki-67 band images were determined on GMS-10 cells at 24 h and also at 48 h, but at 48 h and 72 h reproducible band images were not achieved. These findings suggest that Ki-67 is one of the examples of the proliferation signature in GBM, but further studies are needed to evaluate Ki-67 protein levels with other components of cellular proliferation signaling.

This is the first study to examine SeMet effects on cell growth and death in GMS-10 and DBTRG-05MG cells. Our findings indicate that SeMet induces cell death at high doses, but increases cell proliferation at low doses. Altogether, SeMet appears to be a promising agent for GBM therapy, and the therapeutic potential of SeMet, alone or in combination with conventional treatment agents, warrants additional studies.

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References

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Effects of selenium in glioblastoma multiforme


Sažetak

IN VITRO UČINCI SELENA NA STANIČNE LINIJE HUMANOG GlioBLASTOMA MULTIFORME: PRELIMINARNO ISTRAŽIVANJE

D. Harmanci, Z. Erbayraktar, O. Sayin i G. Akdogan Guner

Glioblastom multiforme (GBM) uzrokuju glijalne stanice podrijetlom iz središnja živčanog sustava i to je najčešći (50%-60%) oblik primarnog tumora mozga. Cilj ovoga istraživanja bio je ispitati in vitro učinke selena na stanice humanog GBM. Rabili smo stanične linije GMS-10 i DBTRG-05MG humanog GBM kao model za ispitivanje ulaska selena u stanicu, stanične proliferacije, citotoksičnosti, fragmentacije DNA i izraženosti proteina Ki-67 u skupini tretiranoj selenometioninom i skupini bez takve obrade. Seleno-L-metionin (SeMet) kao organski izvor selena utjecao je na staničnu proliferaciju i citotoksičnost, što je procijenjeno pomoću testova WST-1 odnosno laktat dehidrogenaze (LDH). Apoptoza je procijenjena fragmentacijom DNA testom ELISA. Izražajnost proteina Ki-67 utvrđena je Western blotingom, dok su mjerenja selena provedena u supernatantima, a lizati primijenom GFAAS. Ovo je prvo istraživanje u kojem su se ispitivali učinci SeMet na staničnu proliferaciju i smrt u stanicama GMS-10 i DBTRG-05MG. Obje stanične linije GBM pokazale su o dozi i vremenu ovisan odgovor na SeMet. Test WST-1 pokazao je da je tretman niskom dozom SeMet (50 i 100 μM) povećao proliferaciju stanica. Rezultati analize unutarstaničnih razina SeMet pomoću AAS bili su sukladni rezultatima testova stanične životnosti i citotoksičnosti. Tretman pomoću SeMet kroz 72 h uzrokovao je povećanu fragmentaciju DNA u objema staničnim linijama. U zaključku, naši rezultati ukazuju na to da SeMet u visokim dozama i u različitim oblicima GBM i u kombinaciji s mogućim sinergičnim spojevima. U svjetlu podataka dobivenih u ovom istraživanju smatramo da bi bilo opravdano daljnja istraživanja usredotočiti na moguću primjenu SeMet kod različitih tipova GBM i u kombinaciji s mogućim sinergističnim spojevima.

Ključne riječi: Glioblastom – terapija; Antikancerogeni lijekovi; Ljudi; Selen; Selenometionin; Stanična proliferacija – učinci lijekova; Stanična smrt – učinci lijekova; antigen Ki-67