ANTICANCER RESEARCH *24*: 2913-2918 (2004)

Selenium Influences the Radiation Sensitivity of C6 Rat Glioma Cells

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Abstract. *Background: A radioprotective effect of low selenium doses on normal tissue and a possible radiosensitization of tumor cells at higher concentrations have been previously suggested. However, systematic experimental and clinical data are scarce. Materials and Methods: C6 cells were cultured for 14 days at different selenite concentrations (0 μM, 2 μM, 3 μM and 3.6 μM) and irradiated with 0 Gy to 20 Gy. Plating efficiency and survival were determined using the colony assay. Results: The overall radiosensitivity was low ($D_{0,control}$=6.1 Gy). Irradiated cells exposed to increasing selenite concentrations showed a lower plating efficiency and, for doses > 2 Gy, a lower survival than the control. For Se concentrations of 0 μM, 2μM and 3 μM, respectively, SF2 amounted to 0.72, 0.48 and 0.46, and SF5 to 0.37, 0.25 and 0.12. Student's t-test yielded a significant difference between the 0 μM and 3 μM curves at 5 Gy (p=0.02) and 10 Gy (p=0.009). Conclusion: Our experiments suggest a radiosensitizing effect of selenite in glioma cells at concentrations of 2-3 μM.*

There has always been interest in agents that are able to influence the radiosensitivity of tumor cells and/or normal tissue to improve the therapeutic ratio, but it has been revived during recent years by the development of various new substances that interact with the radiation sensitivity of cells. One mechanism is the generation or destruction of free radicals. The role of trace elements, especially selenium, in this context is still unclear.

Traditionally, selenium has been associated with radiation protection because of its antioxidant properties. Radiation protection can take place by reactions of free radicals with sulphydryl groups from solute cysteine or peptides and proteins containing cysteine. The most powerful natural antioxidant molecule containing cysteine is glutathione. In recent years, artificial cytoprotectants like amifostine have been developed that also make use of SH groups binding to free radicals. Experimental as well as clinical data support the radioprotective effect of these drugs (1).

There are four known forms of glutathione peroxidase (GPX) containing selenium (2). Selenium glutathione peroxidases catalyze the elimination of hydrogen peroxide as well as organic peroxides (R–O–OH) by the oxidation of GSH. They contain a covalently bound selenium atom in the form of a selenocysteine molecule in their active center (3). The substitution of selenocysteine with normal cysteine at the active site of GPX has been shown to dramatically reduce its enzymatic activity. The forms GPX-1 and GPX-2 are found in the cytosol, while GPX-3 is found in the plasma, and GPX-4 performs special functions in the metabolism of phospholipid hydroperoxides. Both overexpression and knockout models point to an important role of these enzymes in the protection against oxidative attacks (2).

Recently, further selenoproteins have been identified that might possess an antioxidant effect, *e.g.* selenoprotein P (4) and selenoprotein W (5).

Zhong *et al.* (6) have shown that glioma cells in particular possess antioxidant enzymes (superoxide dismutase, catalase) and that their sensitivity to glutathione-modifying drugs like BCNU is correlated to the catalase activity in these cells.

However, not all data on the influence of selenium on the radiosensitivity of cells indicate an antioxidative, *i.e.* radioprotective, effect. Stewart *et al.* (7) found that catalytically active selenium compounds (selenite and selenocystamin) can induce apoptosis and exert a cytotoxic effect by generating superoxide radicals ($O_2^{\bullet -}$). Selenium toxicity in general is thought to be caused by the generation of superoxide. It has been shown to be dose-dependent and

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Key Words: Trace elements, selenium, radiosensitivity, radiotherapy.

Table I. *Toxicity of sodium selenite to C6 cultured cells. nt = not toxic; tox = toxic.*

2 μM	2.4 μM	2.8 μM	3.2 μM	3.6 μM	4 μM	6 μM	8 μM	10 μM	15 μM	30 μM	60 μM
nt	nt	nt	nt	nt	**tox**	**tox**	**tox**	**tox**	**tox**	**tox**	**tox**

limited to compounds that are able to generate the selenide anion ($R\text{-}Se^-$) (8). These results have been confirmed by Lanfear *et al.*, Davis and Spallholz, and Lu *et al.* (9-11), who all detected an induction of apoptosis and DNA strand breaks by selenite and proposed various mechanisms (oxidative stress by oxidation of glutathione, selenite-induced endonuclease activity). Frisk *et al.* (12), who investigated the influence of low doses of various trace elements on human glioma cells, did not find any influence of the presence of selenite on the cells' radiosensitivity.

As we have described before (13), all these data form the theoretical basis for the hypothesis that selenium in its catalytic form, selenite, might exert an influence on the sensitivity of cells and/or tissues to ionizing radiation. While preliminary clinical data suggest that there might be a radioprotective effect of low selenium doses on normal tissues, it still remains unclear whether there is a sensitizing or protective effect on tumor cells when exposed to high, but non-toxic, concentrations of selenite.

We investigated this question *in vitro* by exposing rat glioma cells to different concentrations of sodium selenite and measuring their radiation sensitivity. For most trace elements, there are only few *in vitro* toxicity data for cultured cells (12,14,15). Before we could start to analyze the influence of trace elements on the chemo- and radiosensitivity of cultured C6 cells, we had, therefore, to establish toxicity data for this cell line.

Materials and Methods

Cell culture. The C6 rat glioma cell line (*ATCC*, Rockville, USA), first described by Benda *et al.* (16), was used in the reported experiments. The cells were maintained in DMEM supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 mg/ml streptomycin sulfate, at 37˚C and 5% CO_2.

Toxicity measurements. We started with toxicity measurements to determine the maximum tolerable concentration (MTC). Sodium selenite, which was obtained as a sterile solution, was added to the medium in increasing concentrations, and cells were cultured in this trace element-containing medium for 14 days. Cell survival was assessed qualitatively by light microscopy. The MTC was defined as the maximal concentration where no dead or visibly damaged cells could be detected by microscopic observation after an exposure time of 14 days.

Measurement of doubling-times. The cell doubling-time was measured by plating 10^5 cells and counting the number of cells every 24 hours. The doubling-time was assessed by interpolation of the resulting straight line in a logarithmic coordinate system (taking into account the lag phase at the beginning).

Irradiation. For the main experiments (radiation sensitivity), cells were cultured at four different non-toxic selenite concentrations (control, 2 μM, 3 μM and 3.6 μM) for 14 days (whenever confluence was reached, they were freshly plated).

Before irradiation, cells were freshly plated in the following numbers: 0.5×10^3 (control), 1×10^3 (2 Gy), 2×10^3 (5 and 10 Gy) and 4×10^3 (15 and 20 Gy). (These cell numbers had been shown to yield a reasonable number of non-confluent colonies after irradiation with the corresponding doses in previous experiments.)

The cells were then irradiated using a cobalt-60 machine. Irradiation was carried out from a gantry angle of 180 degrees through a perspex tray with a thickness of 5 mm, selected according to the depth of D_{max} for cobalt-60 gamma radiation. Additionally, a large perspex brick was placed above the cell container to homogenize the dose by increasing backscattering. The radiation doses (calculated at D_{max}) used were 0 Gy (control), 2 Gy, 5 Gy, 10 Gy, 15 Gy and 20 Gy.

Assessment of radiation sensitivity. Cell survival was assessed using the colony forming assay. After seeding the cells and irradiation, a time interval of 6-7 doubling-times (C6: 66-77 hours) was interposed. During this interval, the cells were cultured in the medium described above with the corresponding selenite concentrations. Afterwards, the colonies were stained with methylene blue and all colonies above 50 cells were counted. The plating efficiency was calculated as the number of visible colonies divided by the number of cells plated. The survival rate was defined as the quotient of the plating efficiency for the irradiated cells and the non-irradiated control. All experiments were repeated three times to allow calculation of standard deviation and 95% confidence interval.

The influence of selenium on the radiation sensitivity of the C6 cells was analyzed by comparing the plating efficiency, survival rates and survival curve parameters (SF2, SF5 and D_0) of the trace element-exposed cells and the non-exposed control. Student's *t*-test for unpaired samples was used to statistically compare the survival curves at the different selenite concentrations.

Results

Toxicity. The maximum tolerable concentration (MTC) of sodium selenite amounted to 3.6 μM. For details see Table I.

Doubling-time. After a lag phase of 8 hours, cells started to grow exponentially. The growth curve of the C6 cells yielded a doubling-time t_d of 11 hours. Therefore $6 \times t_d$ amounted to 66 hours, $7 \times t_d$ to 77 hours.

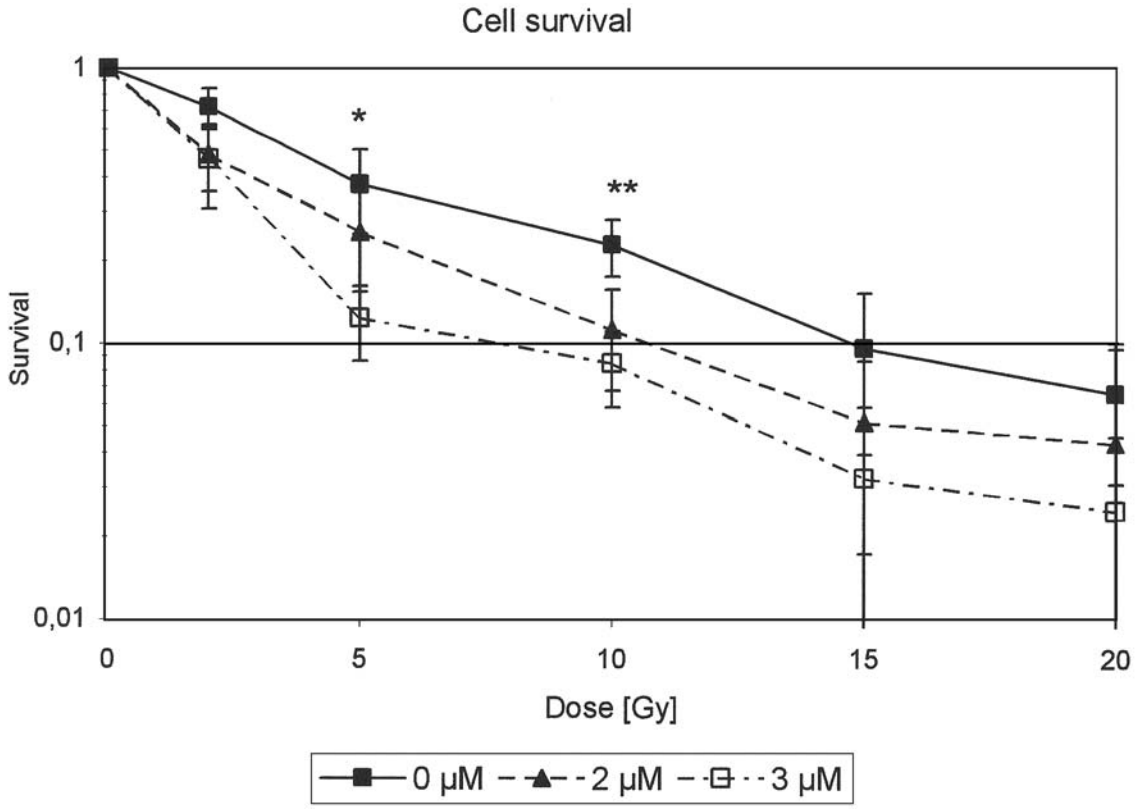

Figure 1. *Logarithmic plot of cell survival at doses between 0 Gy and 20 Gy and different selenite concentrations (0 µM, 2 µM and 3 µM). Bars denote 95% confidence intervals. Asterisks denote significant values for the difference between the 0 µM and 3 µM curves (* p<0.05; ** p<0.01).*

Table II. *Cell survival parameters: surviving fraction at 2 and 5 Gy (SF2/SF5), and mean lethal dose (D_0) for different selenite concentrations. Numbers after "±" denote 95% confidence intervals.*

Selenite concentration	SF2	SF5	D0
0 µM (control)	0.72 ± 0.12	0.37 ± 0.12	6.1 Gy
2 µM	0.48 ± 0.13	0.25 ± 0.09	4.7 Gy
3 µM	0.46 ± 0.16	0.12 ± 0.04	3.8 Gy

Table III. *p values of Student's t-test for unpaired samples by dose for different combinations of selenite concentrations. Asterisks denote significant values (* p<0.05; ** p<0.01).*

Dose	$[Se]_1$	$[Se]_2$	*p*-value
2 Gy	0 µM	2 µM	0.060
	0 µM	3 µM	0.067
5 Gy	0 µM	2 µM	0.207
	0 µM	3 µM	0.020*
10 Gy	0 µM	2 µM	0.032*
	0 µM	3 µM	0.009**
15 Gy	0 µM	2 µM	0.259
	0 µM	3 µM	0.116
20 Gy	0 µM	2 µM	0.065
	0 µM	3 µM	0.120

Radiation sensitivity. Plating efficiency at the different selenite concentrations decreased nearly exponentially with increasing radiation dose. There was only a small influence of selenite concentration on the plating efficiency at 0 Gy (range: 0.72 – 0.81). At 2 to 10 Gy, irradiated cells showed a lower plating efficiency when exposed to selenite concentrations of 2 µM, 3 µM and 3.6 µM than the non-exposed control. The measurements for 3.6 µM showed relatively large variations, possibly due to the start of toxic effects, and were thus omitted from further analysis.

A plot of cell survival with logarithmic scaling of the survival axis can be seen in Figure 1. The cell survival curves at doses > 2 Gy differed markedly between the selenite concentrations and showed lower cell survival with increasing selenite concentrations. The values for SF2, SF5 and D_0 are shown in Table II. The control not exposed to selenite showed a D_0 of 6.1 Gy, which means the C6 cells used in this experiment possessed a rather high radiation resistance (see discussion).

Cell survival at 2 Gy (SF2) and at 5 Gy (SF5) both decreased with increasing selenite concentration to about 1/3 of their values at [Se]=0 μM. D_0 also decreased with increasing selenite concentration.

The *t*-test for unpaired samples yielded a significant difference between the 0 μM and 3 μM curves at radiation doses of 5 Gy (p=0.02) and 10 Gy (p=0.009). The difference at 2 Gy was nearly significant (p=0.07), while at doses ≥ 15 Gy there was no significance (p=0.12). See also Table III.

Discussion

With an SF2 of 0.72 and a D_0 of 6.1 Gy (0 μM control), our results suggest a very low intrinsic radiosensitivity of the C6 rat glioma cells used. This looks rather unusual for mammalian cells; however, similar high D_0 values for this rat cell line have been reported by Takahashi *et al.* (17) and Zhang *et al.* (18). While most human glioma cell lines have a higher radiation sensitivity (D_0=1.58-2.25 Gy) (19), similar SF2 values to ours have also been reported by others (SF2=0.61-0.66) (20,21). For a measurement of the shoulder (N; D_q) we measured too few data points at or below 2 Gy. Graphically the curves appear nearly linear in a logarithmic plot (Figure 1).

Clinical data on the effect of trace elements on tissues exposed to ionizing radiation are still scarce. Tumor patients often have decreased levels of selenium (22). First results suggest that there might be a radioprotective effect of selenium on normal tissues in the clinical setting (23,24). Even a possible protective effect for healthy subjects against cancer has been proposed (22). Some experimental data also suggest a possible superadditive interaction between the radioprotective effects of selenium and the aminothiol amifostine (1).

Sun and coworkers (25) showed that manganese superoxide dismutase and glutathione peroxidase overexpression in Chinese hamster ovary cells were able to reduce their sensitivity to ionizing radiation. Mutlu-Türkoglu and coworkers (26) were able to show that a protective effect of selenium and vitamin E on rat intestine was correlated with an increase of intestinal GPX activity. Their results seem to indicate a radioprotective effect for normal tissue.

On the other hand, for selenium concentrations ≥ 5 μM, several authors reported an induction of apoptosis, DNA strand breaks and a cytotoxic effect by generation of superoxide radicals (7,9-11). These results could support the hypothesis of a synergistic effect between selenite and ionizing radiation, however one has to keep in mind that these data were obtained at toxic selenite concentrations (>= 5 μM).

Frisk *et al.* (12) did not find any influence of the presence of selenite on the radiosensitivity of human glioma cells. This is probably due to the low selenite concentration used in this experiment (0.5 μM, compared to 2–3 μM in our experiments), which is far below the toxic level.

Our experiments indicate a radiosensitizing effect of selenite in irradiated rat glioma cells at non-toxic concentrations of 2 to 3 μM, especially at radiation doses > 2 Gy. If it is true that there is a radiosensitizing effect of selenite on tumor cells – as our results suggest – and a radioprotective effect on normal tissue – as the results of Sun *et al.* and Mutlu-Türkoglu *et al.* suggest –, then selenite might be able to increase the therapeutic ratio for clinical radiotherapy. This hypothesis is further supported by the results of Hehr *et al.* (24), who showed a radioprotective effect of selenium in normal tissue (fibroblasts) but not in tumor cells (squamous cell carcinoma). On the other hand, the question of radiation sensitization or protection could simply be a concentration issue: protection by its antioxidant properties at low concentrations *versus* sensitization by generation of superoxide at high, but non-toxic, concentrations.

Further experiments using other cell lines (tumor as well as normal cells) should also be done to investigate whether there is a sensitization effect in other tumor cell lines and whether this effect extends to normal tissue.

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Received March 3, 2004
Revised June 29, 2004
Accepted June 30, 2004