

L-selenomethionine modulates high LET radiation-induced alterations of gene expression in cultured human thyroid cells

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Abstract. L-selenomethionine (SeM) is emerging as a highly effective protective agent against radiation-induced biological effects. We have shown its protective effect on space radiation-induced death of MCF-10 cells as well as on space radiation-induced transformation of HTori-3 cells. The present study was aimed at elucidation of molecular mechanisms and cellular pathways involved in SeM-mediated radioprotection. Human thyroid epithelial cells (HTori-3 cells), in the presence or absence of SeM, were exposed to a non-toxic or a slightly toxic radiation dose from 1 GeV/n iron ions (10 cGy and 20 cGy, respectively). Total RNA was prepared and changes in gene expression were analyzed using microarray technology. Our analysis has revealed a dramatic effect of SeM on alterations of gene expression caused by space radiation. This study provides a basis for furthering our knowledge about radiation-induced molecular and cellular changes that lead to cellular transformation and death.

Introduction

This report reflects our ongoing investigation into the mechanisms involved in radiation-induced transformation *in vitro* and the suppression of radiation-induced malignant transformation *in vitro* by SeM. The epidemiological and experimental evidence describing ionizing radiation-induced cancer is abundant (1,2). Our daily exposure to background doses of ionizing radiation comes from naturally occurring radioactivity from cosmic rays as well as from radionuclides present in the ground, food, water and building materials. Only occasionally are we exposed to significant doses of

radiation from man-made sources; the main contribution of this source comes from medical treatments. On the other hand, while traveling on space missions, astronauts are exposed to ionizing radiations from an array of particles never encountered on earth due to the shielding effect of the Earth's magnetic field. Of particular importance is radiation from high energy and high atomic number (HZE) particles. Radiation from HZE particles has high linear energy transfer (LET) and, therefore, is considered to be highly effective in causing biological damage. Modification of radiation-induced malignant transformation has been a focus of our research. Studies such as ours advance the concept that radiation-induced malignant transformation or carcinogenesis is not an inevitable progression and can be stopped by the use of non-toxic, non-carcinogenic agents.

Current efforts, focused on the prevention of radiation-induced carcinogenesis in this laboratory, have utilized HTori-3 cells, a human thyroid cell transformation system originally developed by Lemoine *et al* (3) and adapted for studies of radiation transformation by Riches *et al* (4). HTori-3 cells can be transformed by HZE particle radiation (5), and L-selenomethionine (SeM) can prevent HZE particle-induced transformation in HTori-3 cells (5). In both *in vivo* and *in vitro* studies, SeM has emerged as one of the most potent agents evaluated as a countermeasure for HZE particle-induced oxidative stress (5). Non-toxic levels of SeM have been shown to have cancer chemopreventive activity (6-10). SeM is the form of selenium that has been chosen by the National Cancer Institute (NCI) for current studies of selenium as a cancer preventive agent. The largest intervention study using SeM as the cancer preventive agent is a phase-III prostate cancer prevention trial known as the Selenium and Vitamin E Cancer Prevention Trial (11).

The research discussed in this report concerns mechanisms of radiation-induced malignancy and was driven by our hypothesis that radiation is capable of starting an ongoing cellular process (by a high frequency, epigenetic event) that is characterized by a state of genetic instability; a later event or events, presumably genetic, then lead directly to malignant transformation (12,13). The assumptions are that the heritable epigenetic event is a change in gene expression induced by radiation exposure and that L-selenomethionine is capable of

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Table I. Description of the treatment groups.

Treatment group	5 μ M SeM	Radiation dose (cGy)
CT0	No	0
CT10	No	10
CT20	No	20
SEM0	Yes	0
SEM10	Yes	10
SEM20	Yes	20

preventing, suppressing or reversing this change in gene expression. We have used microarray technology to assess the effects of SeM on HZE particle radiation-induced changes in gene expression. HTori-3 cells were exposed to either a non-toxic radiation dose of 10 cGy from iron ions or to a slightly toxic radiation dose of 20 cGy from iron ions. For both radiation doses, SeM had a profound effect on radiation-induced gene regulation.

Materials and methods

Cell culture and treatment with SeM. HTori-3 cells, a human thyroid epithelial cell line, were maintained in DMEM medium (Gibco, Invitrogen) supplemented with 1% glutamine and 10% fetal bovine serum (Atlanta Biologicals). Twenty-four hours prior to radiation exposure, the medium was either supplemented with 5 μ M L-selenomethionine or it was supplement free. At the time of radiation exposure, the cells were confluent.

Exposure to high LET radiation. Exposure to HZE particles was performed at the NASA Space Radiation Laboratory (NSRL) Facility at the Brookhaven National Laboratory, Upton, New York. Cells were exposed to 0, 10 or 20 cGy of 1.0 GeV/n radiation from iron ions.

RNA preparation and microarray hybridization. Six hours post-irradiation, the cells were frozen in denaturing solution (Totally RNA, Ambion) and transported to the University of Pennsylvania, where further steps for isolation of total RNA were taken according to the manufacturer's instructions. Each microarray probe was prepared using 15 μ g of total RNA. First-strand cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies). Following RNA degradation with RNase H and second-strand cDNA synthesis with DNA polymerase I, double-stranded cDNA was extracted using phenol:chloroform:isoamyl alcohol = 25:24:1 (v/v). The double-stranded cDNA template was transcribed and labeled with biotin *in vitro* (IVTA, Enzo Biochem). The resulting cRNA was fragmented and hybridized to U133A Gene Chips (Affymetrix) according to the manufacturer's instructions.

Signal processing and analysis. Gene-level expression values were generated from scanned images (DAT files) using the ViaLog microarray analysis service (VMaXS, <http://www.vialogy.com/>).

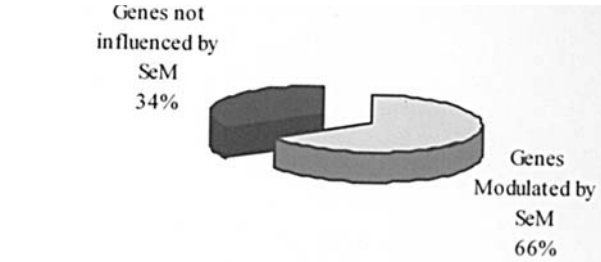


Figure 1. Genes up-regulated by a 10-cGy radiation dose from iron ions.

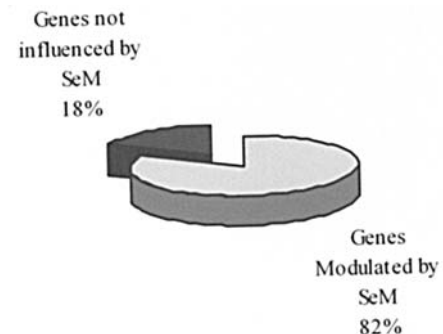


Figure 2. Genes down-regulated by a 10-cGy radiation dose from iron ions.

Gene-level expression values from all 12 microarrays were then quantile normalized (14). Genes differentially expressed as a result of a given treatment were selected as follows. For each gene, four ratios were computed from the replicate hybridizations: treated-A:untreated-A, treated-A:untreated-B, treated-B:untreated-A, and treated-B:untreated-B. The ratios were log₂-transformed and then a score was calculated for each gene using the following formula:

$$score = \frac{\bar{r}_i \sqrt{n_i}}{s_i + 0.5}$$

where n_i is the number of finite log-ratios computed for gene i (if $n_i < 2$, a score was not computed), \bar{r}_i is the mean of the n_i log-ratios, and s_i is the standard deviation of the log-ratios. The genes with the top 300 $|score|$ were selected for further study. EASE over-representation analysis (15) was used to test for Gene Ontology classes that occurred more frequently in a list of genes of interest, significantly regulated genes, than would be expected by chance given the prevalence of a class among all genes assayed.

Results

Monolayer cultures of HTori-3 cells, supplemented with 5 μ M SeM or in non-supplemented medium, were exposed to a 10- or 20-cGy radiation dose from 1 GeV/n iron ions. The cultures were maintained for an additional 6 h post-irradiation, and subsequently trypsinized for preparation of total RNA. Microarray technology was used to analyze changes in gene expression for the 5 treatment groups (Table I) compared to the non-treated group, CT0. To establish the significance of the effect of SeM supplementation on gene expression in irradiated cells, lists of significantly regulated genes were

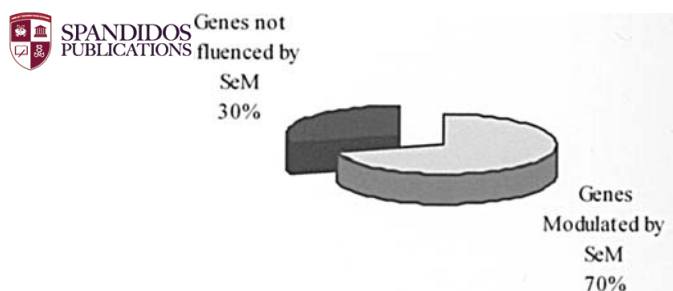


Figure 3. Genes up-regulated by a 20-cGy radiation dose from iron ions.

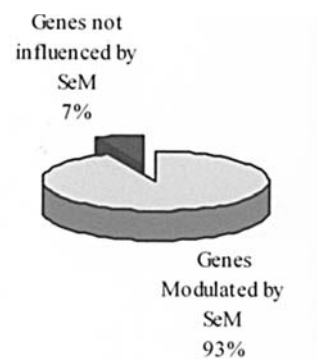


Figure 4. Genes down-regulated by a 20-cGy radiation dose from iron ions.

compared for the shared features. The results of the SEM10 versus CT10 and SEM20 versus CT20 comparisons are shown as pie diagrams in Figs. 1-4. Supplementation with SeM appears to have a profound effect on HZE radiation-induced alterations of gene expression. In HTori-3 cells exposed to a 10-cGy dose of radiation from iron ions, supplementation with SeM modified the differential expression of 66% of up-regulated genes (Fig. 1), and 82% of down-regulated genes (Fig. 2). Similarly, in cells exposed to a 20-cGy dose of radiation from iron ions, supplementation with SeM modified the differential expression of 70% of up-regulated genes (Fig. 3) and 93% of down-regulated genes (Fig. 4). Collectively, these results indicate a large-scale effect of SeM supplementation on regulation of gene expression in HTori-3 cells irradiated with low doses of high LET radiation.

HZE radiation-regulated gene expression which was modifiable by SeM was further analyzed using EASE over-representation analysis. EASE analysis tests for Gene Ontology classes that occur more frequently in a list of genes of interest than would be expected by chance given the prevalence of a class among all genes assayed. The results of

the EASE over-representation analysis indicated that, in HTori-3 cells exposed to a 10-cGy radiation dose from iron ions, SeM abolishes the up-regulation of various structural molecules, including collagen, and molecules with transcription regulator activity (Table II). For the same radiation dose, radiation-induced down-regulation of some components of the ribonucleoprotein complex is modified by SeM (Table III). In HTori-3 cells exposed to a 20-cGy radiation dose from iron ions, SeM abolishes the down-regulation of a significant number of regulators of the cell cycle, including nuclear division components, as well as of molecules involved in transcription from the Pol II promoter (Table IV).

A further analysis using EASE software included significantly regulated genes in HTori-3 cells irradiated in the presence of SeM that were not significantly regulated by SeM alone or by radiation alone. Table V shows that up-regulated genes specific for SEM10 are involved in the negative regulation of cell proliferation and the response to an external stimulus. Also, down-regulated genes specific for

Table II. Over-representation analysis of genes up-regulated by a 10-cGy radiation dose from iron ions in the absence of SeM (CT10) but not in the presence of SeM (SEM10).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score
Collagen	4	104	32	10787	0.0034297
Structural molecule activity	15	105	694	11065	0.0053189
Transcription regulator activity	19	105	1057	11065	0.0092204
Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	38	108	2673	10937	0.0115827
Transcription	28	108	1818	10937	0.0146818
Extracellular matrix	8	104	287	10787	0.0198599
Collagen type VI	2	104	3	10787	0.0283755
Transcription, DNA-dependent	26	108	1756	10937	0.0306572
Protein binding	22	105	1495	11065	0.0366783
Regulation of transcription	25	108	1704	10937	0.0382372
Nucleoside transport	2	108	4	10937	0.0385679
Cell adhesion	11	108	542	10937	0.0391736
Nucleoside transporter activity	2	105	5	11065	0.0461281
Extracellular matrix structural constituent	4	105	88	11065	0.0498686

Table III. Over-representation analysis of genes down-regulated by a 10-cGy radiation dose from iron ions in the absence of SeM (CT10) but not in the presence of SeM (SEM10).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score
Cytosolic large ribosomal subunit	4	50	37	10787	0.000614
Large ribosomal subunit	4	50	48	10787	0.0013197
Cytosol	8	50	384	10787	0.00162
Cytosolic ribosome	4	50	66	10787	0.0032973
RNA binding	7	54	465	11065	0.0230436
Binding	39	54	6414	11065	0.0275012
Ribonucleoprotein complex	6	50	420	10787	0.0409512

Table IV. Over-representation analysis of genes down-regulated by a 20-cGy radiation dose from iron ions in the absence of SeM (CT20) but not in the presence of SeM (SEM20).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score
Nuclear division	7	92	151	10937	0.0015932
M phase	7	92	157	10937	0.0019421
Mitosis	6	92	121	10937	0.0033264
M phase of mitotic cell cycle	6	92	123	10937	0.003569
Cell cycle	14	92	690	10937	0.0044856
Cell proliferation	18	92	1036	10937	0.0047559
Mitotic cell cycle	9	92	329	10937	0.0059962
ATP dependent helicase activity	5	96	102	11065	0.0114239
Transcription from Pol II promoter	10	92	454	10937	0.0130361
Helicase activity	5	96	117	11065	0.0180933
Obsolete cellular component	9	91	433	10787	0.0278196
Chromatin remodeling	3	92	32	10937	0.028891
Non-covalent chromatin modification	3	92	32	10937	0.028891
Nucleus	32	91	2748	10787	0.0358544
Metabolism	60	92	6063	10937	0.0430717
Physiological process	82	92	8954	10937	0.0445714

Table V. Over-representation analysis of genes up-regulated by a 10-cGy radiation dose from iron ions in the presence of SeM (SEM10) but not in the presence of SeM only (SEM0) or by a 10-cGy radiation dose only (CT10).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score	Fisher exact
Negative regulation of cell proliferation	5	83	113	7825	0.0305	0.00683
Response to external stimulus	18	83	1033	7825	0.0371	0.0214

SEM10 are components of the Golgi apparatus and a significant number are involved in regulation of transcription (Table VI). Interestingly, significant numbers of up-regulated

genes specific for SEM20 have roles in the cell cycle and proliferation as well as apoptosis (Table VII). Significant numbers of down-regulated genes specific for SEM20 are



SPANDIDOS PUBLICATIONS Over-representation analysis of genes down-regulated by a 10-cGy radiation dose from iron ions in the presence of M10) but not in the presence of SeM only (SEM0) or by a 10-cGy radiation dose only (CT10).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score	Fisher exact
Golgi apparatus	3	17	243	7038	0.104	0.0193
Transcription regulator activity	4	14	859	8126	0.151	0.0524

Table VII. Over-representation analysis of genes up-regulated by a 20-cGy radiation dose from iron ions in the presence of SeM (SEM20) but not in the presence of SeM only (SEM0) or by a 20-cGy radiation dose only (CT20).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score	Fisher exact
Cell proliferation	18	90	816	7825	0.00962	0.00478
Cell cycle	13	90	542	7825	0.0192	0.00868
Nucleus	33	81	2079	7038	0.0286	0.0199
Apoptosis	9	90	330	7825	0.0336	0.0132
Programmed cell death	9	90	331	7825	0.0341	0.0135
Cell death	9	90	351	7825	0.0456	0.0191
Death	9	90	353	7825	0.0469	0.0197
Structural constituent of cytoskeleton	4	97	75	8126	0.0587	0.0122
Regulation of transcription	20	90	1161	7825	0.0611	0.0385

Table VIII. Over-representation analysis of genes down-regulated by a 20-cGy radiation dose from iron ions in the presence of SeM (SEM20) but not in the presence of SeM only (SEM0) or by a 20-cGy radiation dose only (CT20).

Gene category	List Hits	List Total	Population Hits	Population Total	EASE score	Fisher exact
Vesicle-mediated transport	4	25	194	7825	0.0207	0.00308
Transferase activity	8	26	974	8126	0.0239	0.00892
Transferring phosphorus-containing groups	6	26	584	8126	0.0301	0.00894
Protein amino acid phosphorylation	5	25	395	7825	0.0305	0.00735
Protein kinase activity	5	26	404	8126	0.0332	0.0082
Phosphorylation	5	25	424	7825	0.0382	0.00985

involved in vesicle-mediated transport and some act as protein kinases (Table VIII).

Discussion

Ionizing radiation-induced adverse biological effects, such as death and transformation of cells, have been extensively documented. Of particular concern for the health of astronauts during extended space travel is radiation from HZE particles, which has been shown to increase oxidative stress both *in vivo* and *in vitro*, as well as cytotoxicity and cell transformation *in vitro* (5). SeM has emerged as a potentially useful counter-measure against space radiation-induced adverse biological

effects. To explore the molecular mechanisms involved in the protective effects of SeM, we have used microarray technology to examine changes in gene expression in HTori-3 cells irradiated with 1 GeV/n of iron ions in the presence or absence of selenomethionine.

Changes in gene expression were evaluated in irradiated HTori-3 cells in the absence of SeM (the CT10 and CT20 treatment groups), and in the presence of SeM (the SEM10 and SEM20 treatment groups). A striking difference in radiation-induced gene expression is apparent between SeM-supplemented and -unsupplemented HTori-3 cells. Supplementation with SeM modified the differential expression of 66% of up-regulated and 82% of down-regulated genes in

cells exposed to a 10-cGy radiation dose from iron ions, as well as 70% of up-regulated and 93% of down-regulated genes in cells exposed to a 20-cGy radiation dose from iron ions. The large-scale effect of SeM on radiation-induced regulation of gene expression could have two explanations. SeM plays a major role in antioxidant activities in cells, and part of its action may be due to a scavenging effect, whereby the effective radiation dose reaching cells and eventually causing damage within cells would be significantly reduced. The scavenging effect, however, can not account for the regulation of genes specific for cells irradiated in the presence of SeM but were not differentially regulated in cells that were only supplemented with SeM or only irradiated.

The other mode of action that could explain a large shift in differentially expressed genes would be through modification of one or more transcription regulator activities. From the data presented in this report, a large shift in the expression of various regulators of transcription is evident in irradiated cells when SeM is present. In cells exposed to a non-toxic dose of radiation from HZE particles (10 cGy), the expression of a significant number ($p < 0.01$) of genes involved in the regulation of transcription is up-regulated. In the presence of SeM, however, expression of these genes is not altered upon irradiation with a 10-cGy radiation dose from iron ions. Moreover, a different set of genes involved in regulation of transcription are down-regulated when cells are irradiated with the same dose from iron ions and in the presence of SeM. The present study in HTori-3 cells also indicates that, in cells exposed to a 20-cGy radiation dose from iron ions, SeM supplementation leads to modulation of transcriptional regulation of a significant number of genes involved in the cell cycle, proliferation and death. Future studies will be performed to determine whether this SeM-mediated modulation at the transcriptional level leads to protection against radiation-induced killing.

The results presented here suggest that the mechanism(s) underlying the protective effects of SeM against radiation-induced cell killing and transformation, as well as malignant transformation, can be explored at the level of transcriptional regulation. Our future studies will focus on mechanisms by which ionizing radiation induces transformation of cells. We believe that the use of SeM as a countermeasure will help us to identify molecular targets which are key players in events leading to the malignant transformation of cells.

References

1. National Research Council, Committee on the Biological Effects of Ionizing Radiations: Health Effects of Exposure to Low Levels of Ionizing Radiation, BEIR V. National Academy Press: Washington, DC, 1990.
2. United Nations Scientific Committee on the Effects of Atomic Radiation: Sources and Effects of Ionizing Radiation. UNSCEAR 2000 Report to the General Assembly, with Scientific Annexes. United Nations: New York, 2000.
3. Lemoine NR, Mayall ES, Jones T, Sheer D, McDermid S, Kendall-Taylor P and Wynford-Thomas D: Characteristics of human thyroid epithelial cells immortalized *in vitro* by simian virus 40 DNA transfection. *Br J Cancer* 60: 897-903, 1989.
4. Riches AG, Herceg Z, Bryant PE and Wynford-Thomas D: Radiation-induced transformation of SV40-immortalized human thyroid epithelial cells by single and fractionated exposure to gamma-irradiation *in vitro*. *Intl J Radiat Biol* 66: 757-765, 1994.
5. Kennedy AR, Ware JH, Guan J, Donahue JJ, Biaglow JE, Zhou Z, Stewart JA, Vazquez M and Wan XS: Selenomethionine protects against adverse biological effects induced by space radiation. *Free Radic Biol Med* 36 (2): 259-266, 2004.
6. Li JY, Taylor PR, Li B, Dawsey S, Wang GQ, Ershow AG, Guo W, Liu SF and Yanglast CS: Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality among adults with esophageal dysplasia. *J Natl Cancer Inst* 85: 1492-1498, 1993.
7. Reddy BS, Rivenson A, El-Bayoumy K, Upadhyaya P, Pittman B and Rao CV: Chemoprevention of colon cancer by organo-selenium compounds and impact of high- or low-fat diets. *J Natl Cancer Inst* 89: 506-512, 1997.
8. Nelson MA, Porterfield BW, Jacobs ET and Clark LC: Selenium and prostate cancer prevention. *Semin Urol Oncol* 17: 91-96, 1999.
9. Khuri FR and Lippman SM: Lung cancer chemoprevention. *Semin Surg Oncol* 19: 100-105, 2000.
10. Clark LC, Combs GF Jr, Turnbull BW, Slate EH, Chalker DK, Chow J, Davis LS, Glover RA and Grahamlast GF: Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. *JAMA* 276: 1957-1963, 1996.
11. Klein EA, Thompson IM, Lippman SM, Goodman PJ, Albanes D, Taylor PR and Coltman C: SELECT: the next prostate prevention trial. Selenium and Vitamin E Cancer Prevention Trial. *J Urol* 166: 1311-1315, 2001.
12. Kennedy AR, Fox M, Murphy G, Little JB: Relationship between x-ray exposure and malignant transformation in C3H10T1/2 cells. *Proc Natl Acad Sci USA* 77: 7262-7266, 1980.
13. Kennedy AR, Cairns J and Little JB: Timing of the steps in transformation of C3H10T1/2 cells by x-irradiation. *Nature* 307: 85-86, 1984.
14. Bolstad BM, Irizarry RA, Astrand M and Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19 (2): 185-193, 2003.
15. Hosack DA, Dennis G Jr, Sherman BT, Lane HC and Lempicki RA: Identifying biological themes within lists of genes with EASE. *Genome Biol* 4 (10): R70, 2003.