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Enhanced lung cancer cell killing by the combination of selenium and ionizing radiation

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Abstract. Selenium has been associated with anticancer activity by affecting multiple cellular processes. We reasoned that the simultaneous modulation of multiple radioresponse regulators by selenium should increase radiosensitivity if selenium is combined with radiation in cancer therapy. Therefore, we explored the possibility of whether we could obtain an enhancement of radiosensitivity by the combination of selenium and ionizing radiation. We used two human lung cancer cell lines, NCI-H460 and H1299, as well as a human diploid lung fibroblast, WI-38, as the normal cell counterpart. The combined treatment of the cancer cell lines with Seleno-methionine and ionizing radiation resulted in increased cell killing as assessed by clonogenic survival assay whereas it had little effect on the normal diploid WI-38 cells. The increased radiosensitivity in the cancer cells was correlated with the attenuation of the key proteins involved in either cell survival signaling [Akt, EGFR (epidermal growth factor receptor), ErbB2 and Raf1] or DNA damage response (Mre11, Rad50, Nbs1, Ku80, 53BP1 and DNAPK). The attenuation of the proteins by the selenium compound was possibly caused by the effect on transcription and on protein stability since selenium treatment decreased both the RNA transcript and the protein stability of EGFR and DNAPK. By contrast, Seleno-L-methionine had no effect on the protein profile of a normal diploid fibroblast which is consistent with an intact radiosensitivity. These data provide possible clinical applications, as selenium selectively enhanced the radiosensitivity of the tumor cells whereas that of the normal cells was unaffected. Moreover, the selective decrease of cell proliferation signaling in tumor cells but not in normal cells should facilitate the repopulation of normal cells required for healing during radiation therapy. On the whole, the results suggest that the cancer preventive activity of selenium can be combined with ionizing radiation to improve the control of lung cancer.

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Introduction

Selenium has been associated with cancer-preventive effects (1). Human epidemiological studies have shown that an inverse relationship exists between selenium intake and the risk of cancer (2). In support of this observation, it has been shown that a selenium supplement is capable of reducing the incidence of lung, prostate, and colon cancer (3).

Molecular targets for the cancer preventive effects of selenium have been delineated in several cellular systems. Analysis of the gene expression profiles in several cancer cells revealed that the selenium-induced growth inhibition is associated with the modulation of the cell cycle, apoptosis and signaling (4,5). Although the targets are variable by cell types, it is clear that selenium affects not just one target, but a multitude of targets involved in diverse cellular functions. The targeting of multiple molecules should be able to enhance the impact of selenium, thereby making it difficult for the cancer cells to evade its cancer-preventive effects.

Agents that affect multiple targets have been used to enhance sensitivity to radiation. Given the diverse survival signaling pathways and molecules involved in radiation response, the targeting of multiple radioresponse regulators simultaneously, a so-called multitarget approach, should enhance radiosensitivity substantially more than the targeting of only one molecule (6). Therefore, we wanted to evaluate whether selenium has the capacity to modulate radiosensitivity in a similar multitarget approach and what radioresponse regulators are potentially involved in the process.

A few studies have suggested that selenium is associated with the modulation of radiosensitivity (7,8). However, the molecular causes remain ambiguous. To address these issues, we exposed two lung cancer cell lines and a normal diploid fibroblast to Seleno-L-methionine (SeMet), which is the selenium compound used in the SELECT (selenium and vitamin E chemoprevention trial) (9), alone or in combination with radiation. Thereafter, we evaluated the effects of SeMet on

the radiosensitivity of the cell lines, and on the levels of two different classes of radioresponse-regulatory proteins which are involved either in cell survival signaling or in DNA damage response.

Materials and methods

Cell lines and treatment. Two human lung cancer cell lines (NCI-H460 and NCI-H1299) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Each cell line was grown in RPMI-1640 (Life Technologies, Inc., Rockville, MD, USA) containing 5 mM glutamate and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and maintained at 37˚C in an atmosphere of 5% CO_2 and 95% room air. The normal diploid human fibroblast cell line WI-38 was also obtained from ATCC. WI-38, which was originally derived from normal embryonic lung tissue, was grown in the same medium conditions as above except for the addition of 1XMEM non-essential amino acid (Sigma, St. Louis, MO, USA) and used for experiments in the passages between 20 and 25. Seleno-L-methionine (SeMet, Sigma), was dissolved in DMEM to a stock concentration of 100 mM and stored at -20˚C. The effect of selenium on cell proliferation was measured by MTT assay (Sigma) following the manufacturer's protocol. The cell cultures were irradiated using a ^{137}Cs gamma-ray source (Atomic Energy of Canada Ltd., Mississauga, Canada) at a dose rate of 3.81 Gy/min.

Clonogenic assay. The colony-forming ability after the treatment of each cell line was compared using clonogenic assay as described previously (10). Briefly, the cultures were trypsinized to generate a single cell suspension, and 500 cells were seeded into 6-cm tissue culture plates. After giving the cells time to attach (24 h), SeMet was added at specified concentrations, and the plates were irradiated 24 h later. Immediately after irradiation, the SeMet-containing media were aspirated, and fresh media were added. Ten to 14 days after seeding, the colonies were stained with trypan blue, the number of colonies containing at least 50 cells was determined, and the surviving fractions were calculated.

Immunoblot analysis. Cells were scraped into PBS, centrifuged, and the cell pellet was resuspended in 3 volumes of lysis buffer [40 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Nonidet P-40, and protease inhibitors] and the proteins were extracted. Immunoblot analysis was then performed as described previously (10). The antibodies to cRaf-1, Akt, EGFR (epidermal growth factor receptor), ErbB2, Mre11, Nbs1, Rad50, DNA-PK, Ku70, Ku80 and 53BP1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies to beta-actin were obtained from Sigma. The blots were developed by a peroxidase-conjugated secondary antibody and the proteins were visualized by ECL procedures (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's protocol.

Cell cycle phase analysis. The evaluation of the cell cycle phase distribution was performed using flow cytometry. The treatment protocols were essentially the same as those in the clonogenic survival experiments, except that the cells were initially seeded into 10-cm dishes. All the cultures were subconfluent at the time of collection. The cultures were collected for fixation, stained with propidium iodide, and analyzed as described previously (11).

RT-PCR assay and measurement of protein half-life. The level of the EGFR and DNAPK transcripts was measured by RT-PCR analysis. After treating the NCI-H460 cells with specified concentrations of SeMet, total RNA was isolated and used in RT-PCR assay. The following oligonucleotide primers were used to amplify the transcripts (we used 32 cycles of 94˚C, 30 sec; 55˚C, 50 sec; 72˚C, 30 sec):

EGFR (L primer: 5'-CCACCAGAGTGATGTCTGGA GCTA-3', R primer: 5'-GGCACGGTAGAAGTTGGAGTC TGT-3'), DNAPK (L primer: 5'-CAACTTCAGATCCAG CGGCTAACT-3' R primer: 5'-GGCAAGGACAGAAGA AAGGTCAAA-3'). GAPDH was used as a control. GAPDH (L primer: 5'-CATGGAGAAGGCTGGGGCTCATTT-3' R primer: 5'-CGCCAGTAGAGGCAGGGATGATGT-3').

To measure the half-life of the proteins, the NCI-H460 cells were treated with specified doses of SeMet for 24 h. At the end of the SeMet treatment, cycloheximide (80 μg/ml) was added and the cell were incubated further. At the specified time points, the cells were harvested and extracts were made for Western blot analysis. The densitometer measurement of the protein band intensity was graphed to determine the half-life of the proteins.

Results

We used the selenium compound, SeMet to evaluate whether it influences the radiosensitization of the two lung cancer cell lines, NCI-H460 and NCI-H1299. The WI-38 cell line was used as a normal diploid lung cell line control.

As an initial effort, we evaluated SeMet's effect on cell viability and growth following 24-h exposure of the cells to SeMet alone (Fig. 1). The cell lines showed different sensitivities to SeMet as determined by clonogenic assay (Fig. 1A). Among the cancer cell lines tested, the NCI-H460 cells were less sensitive to the treatment than the NCI-H1299 ones. About 70% of the plated cells of NCI-H460 survived whereas 50% of the NCI-H1299 cells formed colonies. Compared to the cancer cell lines, the normal fibroblast WI38 was more resistant with >80% of the cells being viable following the treatment with 200 μM SeMet (Fig. 1A).

Since selenium has been reported to have anti-growth properties (4,5), we next determined how the cell growth was affected by the 24-h SeMet treatment by using MTT assay (Fig. 1B-D). The cells were washed and fed with normal growth media following treatment with 0, 50, 100 and 200 μM SeMet for 24 h and incubated further for up to 72 h for MTT assay. Consistent with a previous report (12), the SeMet treatment of the cancer cell lines caused a dose-dependent decrease in cell growth (Fig. 1B and C). When the cell growth was measured after incubating for 24 h following the 200 μM SeMet treatment, the NCI-H460 cells showed a 50% decrease in growth compared to the vehicle only treatment whereas 50 μM SeMet caused only about a 10% reduction (Fig. 1B). A longer incubation of the SeMet treated-NCI-H460 cells decreased the growth further. After 72-h

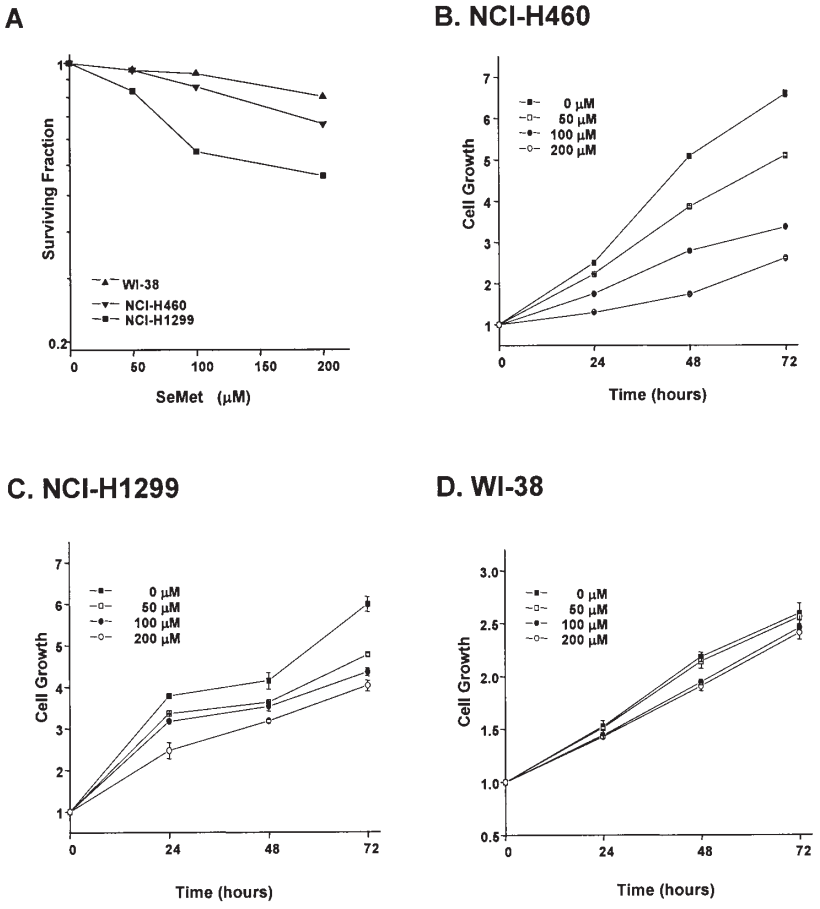

Figure 1. Cell survival and growth curves for the three cell lines, NCI-H460, NCI-H1299, and WI-38, following the exposure of the cells to Seleno-L-methionine (SeMet) alone (mean ± SE). (A) Cell survival after exposure to SeMet. The cells were exposed to various concentrations of SeMet for 24 h, and the colony-forming ability was measured by clonogenic assay. Values represent the mean ± the SE from four independent experiments. (B-D) MTT assay was used to measure the cell growth after exposure to 0, 50, 100 and 200 μM SeMet. MTT assay was performed as described in Materials and methods. (B) The lung cancer cell line, NCI-H460. (C)The lung cancer cell line, NCI-H1299. (D) The normal diploid lung fibroblast, WI-38.

incubation following the 200 μM SeMet treatment, the NCI-H460 cell growth was inhibited by 60%. The NCI-H1299 cell line showed a similar decrease in cell growth although the degree of cell growth inhibition varied (Fig. 1C). The growth of the WI38 normal cells was hardly affected by the SeMet treatment (Fig. 1D). These results suggest that the decreased cell growth by SeMet in the cancer cell lines may partly account for the decreased clonogenic cell survival.

SeMet is likely to affect not just one critical target, but multiple radioregulatory proteins as is evident by the analysis of gene expression by microarray methods (4,5). Multiple targeting by selenium could result in the enhancement of radiosensitivity. To explore this possibility, we examined the levels of radio-regulatory protein targets by Western blot analysis. We selected targets for radiation resistance from two groups of proteins, i.e. proteins involved in cell proliferation signaling such as AKT, EGFR, ErbB2 and Raf1 (Fig. 2) and proteins in DNA double strand break repair (DSBR) including Mre11, Rad50, Nbs1, Ku proteins, 53BP1 and DNAPK (Fig. 4).

The lung cancer cell lines, NCI-H460 and NCI-H1299, showed a concentration-dependent decrease in the levels of Akt, EGFR, ErbB2 and Raf1 (Fig. 2A and B). The decrease in protein levels was significant at 100 μM and 200 μM SeMet. The control protein, beta actin, was unaffected by the treatment in each cell line. The protein levels in the normal fibroblast, WI-38, were unaffected by the treatment (Fig. 2C). Based on the protein levels, we found that the two lung cancer cell lines, H460 and H1299, were sensitive to the SeMet treatment whereas the WI-38 cell line was unaffected. Moreover, the results suggest that the decreased levels of cell proliferation signaling molecules in the lung cancer cell lines might result in an increased radiosensitivity.

To assess whether SeMet treatment enhances the sensitivity of the tumor cells to radiation-induced cell death, the cell lines were exposed to SeMet and incubated for 24 h. After irradiation, the colony-forming ability of each cell line was determined. The combined treatment increased the radiosensitivity of the lung cancer cell line, NCI-H460, compared with that of radiation only (Fig. 3A). The survival decrease was supra-additive in the combined treatment, that is, a greater decrease in cell viability could be observed than the sum of each treatment alone. Since 4 Gy radiation alone resulted in a 50% survival and 70% with 200 μM SeMet treatment alone, we expected a 35% (0.5x0.7) survival with the combined treatment. However, the combined treatment resulted in 21% survival indicating the enhancement of radiosensitivity. The exposure of another lung cancer cell line,

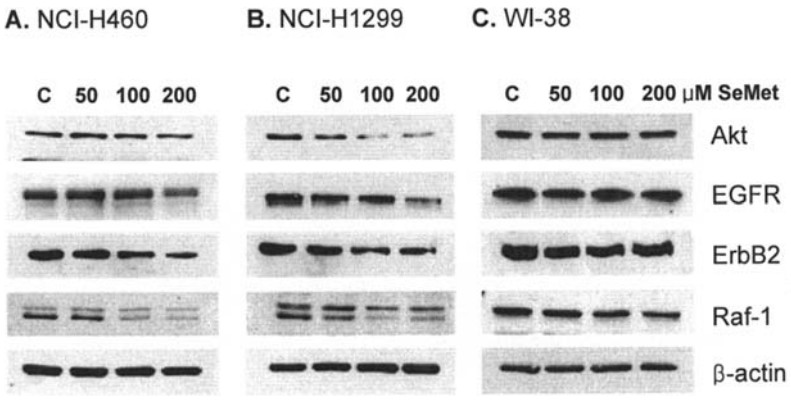

Figure 2. The levels of the radioresistant-associated proteins by the Seleno-L-methionine (SeMet) treatment: Western blot analysis of Akt, EGFR, ErbB2, Raf1 and beta actin as a control in different cell lines. Immunoblots were generated from each cell line after 24-h exposure to the specified SeMet concentrations. Each blot is representative of three independent experiments. (A) The lung cancer cell line, NCI-H460. (B) The lung cancer cell line, NCI-H1299. (C) The normal diploid lung fibroblast, WI-38.

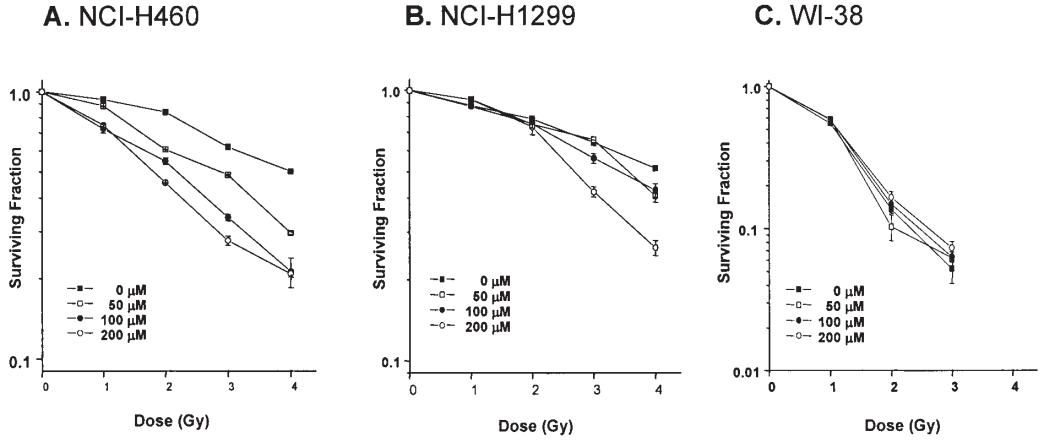

Figure 3. The effect of Seleno-L-methionine (SeMet) on cellular radiosensitivity. The cells were exposed to specified concentrations of SeMet for 24 h, irradiated with graded doses of gamma-rays, rinsed, and fed with fresh growth media. The colony-forming efficiency was determined and the survival curves were generated after normalizing for cell killing by SeMet alone. Values represent the mean ± SE from four independent experiments. (A) The lung cancer cell line, NCI-H460. (B) The lung cancer cell line, NCI-H1299. (C) The normal diploid lung fibroblast, WI-38. Note the different scale in the Y-axis of the WI-38 survival curve (C).

NCI-H1299, led to a similar result with an enhanced radiosensitivity at 100 and 200 μM SeMet-treated cells (Fig. 3B).

The reduced lung cancer cell survival was correlated with the decreased levels of radio-regulatory proteins such as AKT, EGFR, ErbB2 and Raf1 with a prominent decrease at 100 and 200 μM SeMet concentrations. The correlation between cell survival and the levels of the radio-regulatory proteins suggests that the down-regulation of the proteins by SeMet treatment could underlie the enhancement of radiosensitivity by the combined treatment in the lung cancer cell lines.

A longer than 24-h incubation of the cell lines with SeMet caused an even more severe decrease in the levels of the radioresponse regulatory proteins with a proportionally increased radiosensitivity (data not shown).

The therapeutic potential of SeMet as a radiation modifier will depend on a selective increase in the radiosensitivity of tumor cells over normal cells. To assess this potential, we evaluated the effects of SeMet on the radiosensitivity of the normal diploid human fibroblast WI-38 cells derived from a human embryonic lung. The treatment protocol was the same as the one used for the tumor cell lines. Monolayer cultures of exponentially dividing WI-38 cells were exposed to various concentrations of SeMet, irradiated and the clonogenic survival was determined.

It is notable that the radiosensitivity of WI-38 normal diploid fibroblast cells is considerably greater than that of the tumor cell lines (compare the control survival curves in Fig. 3). This enhanced radiosensitivity of the normal diploid fibroblast (or the resistance of the tumor cell lines) is consistent with a previous report (6). The radiosensitivity of the normal diploid fibroblast WI-38 cells was unaffected by the SeMet pretreatment (Fig. 3C). The difference in the cell viability of WI-38 between the SeMet-treated and the untreated following irradiation was marginal. The results indicate that SeMet exposure resulted in the selective enhancement of radiosensitivity in the human lung cancer cell lines evaluated, whereas it has no effect on that of the normal lung cell line.

Because the cell cycle distribution can also affect the radiosensitivity of a particular cell line (13), we determined the cell cycle distribution of the cell lines using FACS (Fluorescence-Activated Cell Sorter) analysis (Table I). The cell lines were

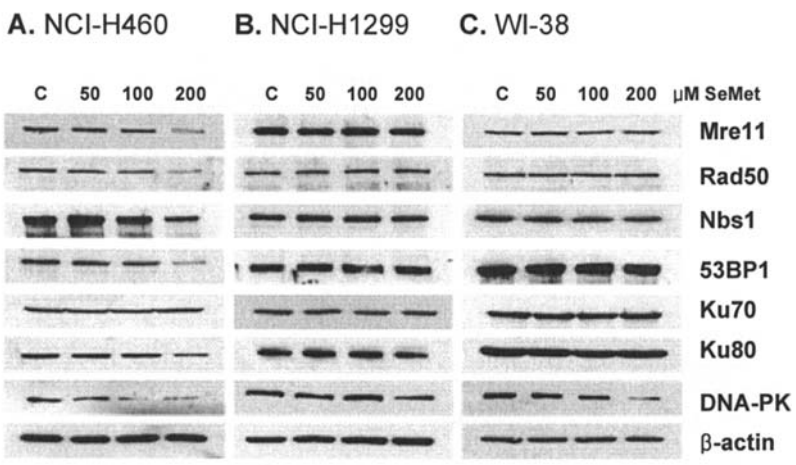

Figure 4. The levels of the proteins involved in DNA damage response or double strand break repair by the Seleno-L-methionine (SeMet) treatment: Western blot analysis of Mre11, Rad50, Nbs1, 53BP1, Ku70/80, DNA-PK, and beta actin as the control. Immunoblots were generated from each cell line after 24-h exposure to the specified SeMet concentrations. Each blot is representative of three independent experiments. (A) The lung cancer cell line, NCI-H460. (B) The lung cancer cell line, NCI-H1299. (C) The normal diploid lung fibroblast, WI-38.

Table I. The effect of SeMet on the cell cycle phase distribution and cell death.

	NCI-H460		NCI-H1299		WI-38	
	0 (μM)	200 (μM)	0 (μM)	200 (μM)	0 (μM)	200 (μM)
SubG1	1.18	3.26	0.57	1.22	2.83	2.60
G1	48.46	43.02	49.71	64.09	44.95	29.97
S	14.37	5.75	22.61	14.08	12.69	11.21
G2/M	29.68	42.25	23.32	18.08	35.12	51.13

The tumor cell lines were exposed to the control vehicle (0) or a radiosensitizing concentration of Seleno-L-methionine (SeMet) (200 μM) for 24 h. The cells were then collected and subjected to FACS analysis to measure the distribution of the cell cycle phase and cell death (subG1 fraction). This is a typical result from repeated cell cycle analysis.

exposed to either 0 or 200 μM SeMet for 24 h and the distribution of the cell cycle phase was examined. The cells showed variable patterns of distribution in the cell cycle phase (Table I). The SubG1 population in the cell lines was negligible, which indicates that no significant apoptotic cell death occurred by the SeMet concentration. The G1 phase of the H1299 cells increased, whereas that of the NCI-H460 and WI-38 cells decreased. A slight decrease in the S phase could be observed in each of the tumor cell lines. The NCI-H460 and WI-38 cells showed a considerable increase in the radiosensitive G2/M phase. Given the results, the redistribution of the cell cycle phase by the SeMet treatment may partly account for the increased radiosensitivity of the cancer cells.

We also examined whether SeMet affects proteins involved in DNA double strand break (DSB) repair, especially proteins involved in NHEJ (non-homologous end joining) because the lack or dysfunction of the proteins has been associated with hypersensitivity to ionizing radiation (14,15). We examined the protein levels of Mre11, Rad50, Nbs1, Ku70, Ku80, 53BP1 and DNAPK (Fig. 4). These proteins decreased in a cell type-dependent fashion. For example, Mre11, Rad50, Nbs1, Ku80, 53BP1 and DNAPK were attenuated in the H460 cells upon exposure to SeMet (Fig. 4A). The reduction of the proteins was significant at 200 μM SeMet. No significant changes could be observed in the H1299 cell lines (Fig. 4B).

The degree of decrease in the protein levels was variable in each individual protein. For example, in the NCI-H460 cells, the reduced DNAPK was evident from 100 μM and 200 μM SeMet whereas Mre11, Rad50, Nbs1, 53BP1 and Ku80 showed a significant decrease at 200 μM (Fig. 4A). The normal WI-38 cells showed a decreased DNAPK at a higher dose (200 μM) of SeMet whereas the other proteins were not affected (Fig. 4D). The level of Ku70 in all the cell lines used was unaffected by the treatment.

Given the results, it is suggested that in certain cell lines such as NCI-H460, the proteins involved in DNA damage response are affected by the SeMet treatment and are likely to contribute to the modulation of radiosensitivity. Unrepaired DNA damage caused by the insufficient amount of key proteins involved in DNA damage repair could lead to a decreased cell viability.

In order to elucidate the mechanisms of the SeMet effect on attenuating the expression of the radioresponse regulators, we examined the transcript and protein stability of the affected molecules in the NCI-H460 cells (Fig. 5). SeMet attenuated both the levels of the transcripts and the protein half-life, indicating that SeMet had an effect on transcription and protein stability (Fig. 5). The SeMet treatment reduced the EGFR and DNAPK transcription whereas the GAPDH control remained intact (Fig. 5A). The protein stability of EGFR and DNAPK was significantly decreased by SeMet as revealed by the measurement of their half-life (Fig. 5B and C). On the whole, these data suggest that the SeMet treatment

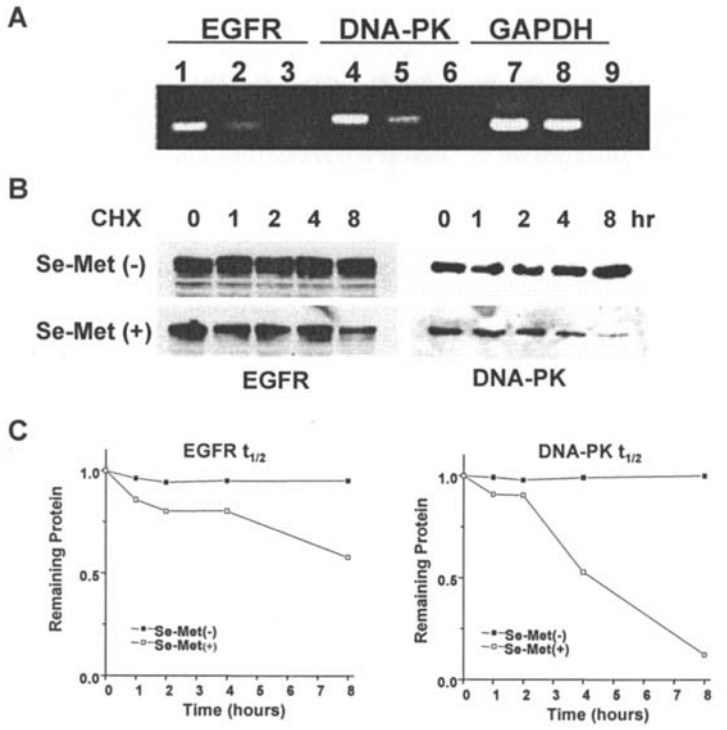

Figure 5. The effect of Seleno-L-methionine (SeMet) on the transcription and protein stability of EGFR and DNAPK. (A) RT-PCR analysis for EGFR and DNAPK gene expression. NCI-H460 cells were exposed to SeMet for 24 h and total RNA was isolated to determine the level of the transcripts for EGFR and DNAPK by RT-PCR assay. GAPDH was used as the control. Lanes 1, 4 and 7 are the RT-PCR results of the untreated cells; lanes 2, 5, and 8 are those of the SeMet treated samples; lanes 3, 6, and 9 are (-) reverse transcriptase reactions which serve as the negative control. (B) The measurement of EGFR and DNAPK protein half-life. The effect of the SeMet exposure on the protein stability of EGFR and DNAPK was determined by measuring the half-life of each protein. The NCI-H460 cells were exposed to SeMet for 24 h and cycloheximide was added to block protein synthesis. After the specified time interval, the levels of the proteins were detected by immunoblot analysis. This result is typical of two separate experiments. (C) A graphical representation of (B). The band intensity from (B) was measured by a densitometer and graphed to show the protein stability. $t_{1/2}$, protein half life.

enhanced the radiosensitivity of the lung cancer cells by attenuating the transcription and protein stability of certain radioresponse regulators involved in either cell proliferation signaling or DNA damage repair.

Discussion

The use of radiation with an agent that targets a multitude of radio-regulatory molecules has been used to increase tumor radiosensitivity. Given the diversity of cell survival signaling and molecular cross talks as well as heterogeneity in gene expression, the simultaneous attenuation of multiple radio-response regulators should increase radiosensitivity. Such a multitarget approach has been demonstrated with the Hsp90 inhibitor, 17AAG. The combined use of the Hsp90 inhibitor with radiation enhanced tumor cell killing by selectively targeting Hsp90 client proteins such as Raf1, ErbB2, and Akt (6).

Since selenium has been associated with cancer prevention by affecting multiple cellular pathways, we evaluated SeMet for its possible use in a multitarget-based approach to enhance radiation sensitivity.

Incubation of the cells with SeMet for 24 h resulted in a concentration-dependent decrease both in cell viability and growth (Fig. 1). It has been reported that the growth inhibitory effect of selenium on the tumor cell lines is attributable to a combined effect on the cell cycle and apoptosis (4,16,17). Apoptotic cell death, as represented by the proportion of cells in the subG1 phase among the cell lines evaluated in our report, was minimal, suggesting that apoptosis is not responsible for the inhibition (Table I). Cell growth inhibition was evident with different degrees in the cell lines evaluated that harbor different genetic backgrounds suggesting that certain differences in genotype such as p53 are not required for the inhibition (Fig. 1B-D).

The evaluation of the radioresponse regulatory proteins revealed a cell type- and SeMet concentration-dependent decrease. These proteins are associated with the modulation of radiosensitivity and are involved in either cell proliferation signaling such as AKT, EGFR, ErbB2 and Raf1 (Fig. 2) or in DNA damage response including Mre11, Rad50, Nbs1, Ku proteins, 53BP1 and DNAPK (Fig. 4). As these proteins have a role in radioresistance, the decreased protein levels by the SeMet treatment resulted in the enhancement of radiosensitivity (Fig. 3). The SeMet-mediated enhancement in radiation-induced cell killing occurred in both the human lung cancer cell lines, NCI-H460 and H1299. Considering the diverse genetic background of these cells including the status of p53, the data suggest a general nature of SeMet to enhance radiosensitivity.

AKT, EGFR, ErbB2 and Raf1 are proteins involved in cell survival signaling and conversely, a reduction in the activity or levels of the proteins has been associated with radiosensitization. AKT, which is a kinase downstream of

nositide-3 kinase (PI3K) promotes cellular survival resistance. Activated AKT in non-small cell lung cancer cells has been reported to promote resistance to radiation therapy (18). Therefore, inhibitors targeting PI3K signaling such as LY294002 and wortmannin were used to enhance radiation sensitivity (19,20).

Raf, which is downstream of RAS and constitutes the Raf-MEK-ERK pathway, has also been linked to tumor radioresistance (19). The activated Raf1 gene is frequently found in pancreatic cancer and its overexpression has been associated with resistance to radiation therapy in patients with HNSCC (head and neck squamous cell carcinoma) (21,22). Conversely, the inhibition of Raf1 translation by an antisense oligonucleotide increased radiosensitivity confirming the association of Raf1 with radioresistance (23).

With regard to the EGFR family, its expression is elevated in a variety of tumors including breast, lung, colorectal, and prostate cancer (24). It has been shown that the addition of the exogenous epidermal growth factor to cell cultures can render cells radioresistant *in vitro* (25). By contrast, the inhibition of EGFR signaling by a monoclonal antibody or tyrosine kinase inhibitors of EGFR resulted in the radiosensitizing effect (26,27).

Assessment of the correlation between the levels of AKT, EGFR, and the Raf1 proteins, and radiosensitivity in lung cancer cell lines revealed that SeMet enhanced radiosensitivity with a corresponding decrease in the protein levels. The three proteins involved in cell survival signaling decreased in a SeMet concentration-dependent manner in the NCI-H460 and H1299 lung cancer cell lines (Fig. 2A and B). In order to identify additional radio-regulatory proteins that are affected by SeMet, we evaluated Mre11, Rad50, Nbs1, Ku70, Ku80, 53BP1 and DNAPK. These proteins are involved in DNA damage response and have been associated with the modulation of radiosensitivity. (15).

The NCI-H460 cells showed decreased levels of the proteins involved in DNA damage repair suggesting that insufficient amounts of these repair proteins could lead to the incomplete repair of DNA damage caused by ionizing radiation and contribute to the enhancement of radiosensitivity by the combined treatment. Since the NCI-H1299 cancer cell line exhibited little change in the levels of the DNA repair proteins by the SeMet treatment, these factors can not be what caused the increased radiosensitivity. Rather, the modulation of the cell survival or proliferation signaling could be what caused the response in the NCI-H1299 lung cancer cell line.

Each cell line showed a different profile of the attenuated proteins by the SeMet treatment. Although each one of the proteins is associated with radioresistance, it is difficult to establish the causal relationship between the number of the attenuated proteins and the degree of enhancement in cell killing. Additionally, based on the data presented, it is not possible to assess the degree of contribution by each of the proteins to the overall radiosensitivity. However, collateral damage to the proteins involved in the different pathways should synergistically enhance radiation sensitivity by the combined treatment. For example, the concomitant decrease of cell survival signaling and the DNA repair protein could lead to enhanced radiosensitivity. Our results show that the SeMet treatment affected at least one protein from each of the pathways in the lung cancer cell line, NCI-H460 (Fig. 2A and 4A). The ability of SeMet to target a multitude of molecules involved in DNA damage response could be especially useful for the development of multitarget-based radiotherapy.

SeMet had no effect on the radiosensitivity of the WI-38 normal diploid fibroblast cell line. This is desirable for a possible clinical application, as SeMet selectively enhanced the radiosensitivity of the tumor cells whereas that of the normal cells was unaffected. Moreover, the selective downregulation of cell proliferation signaling in tumor cells but not in normal cells should facilitate the repopulation of normal cells required for healing during radiation therapy. Indeed, the selective modulation of the efficacy of anticancer drugs by selenium has been reported. The combined use of anticancer drugs with selenium had a higher curing rate by selectively protecting the normal tissues while curing mice with human tumor xenografts (28).

The effect of the selenium compounds on radiation response has been ambiguous. Selenium and/or vitamin E pretreatments ameliorated irradiation-induced rat intestinal injury (29). By contrast, selenium had a radiosensitizing effect when C6 rat glioma cells were treated with selenite (7). Although the mechanisms that underlie these contrasting results of the selenium effects are still open to speculation, a dose-dependent mechanism has been suggested by which a radioprotection at low selenium doses and a radiosensitization at higher concentrations are achieved. In line with this suggestion, the sodium selenite treatment of C6 rat glioma cells had a radiosensitizing effect only at concentrations higher than 2 μM (7).

We observed a radioprotective effect at lower concentrations of SeMet around 20 μM (data not shown) whereas higher concentrations of SeMet ranging from 50 to 200 μM had a radiosensitizing effect. Notably, 100 and 200 μM SeMet had a significant radiosensitizing effect depending on the cell lines. Moreover, our data suggest that a possible mechanism for the radiosensitizing effect of selenium is via the downregulation of the radioresponse regulators. The key protein molecules involved in cell proliferation or DNA damage response were selectively downregulated by the selenium treatment thus providing an increased radiosensitivity.

Although this investigation illustrates the potential use of selenium in radiation therapy, the proper dose and effective forms of selenium have to be determined in order for it to be of use in a potential clinical application. SeMet and other selenium compounds are metabolized in the liver into a variety of metabolites including monomethylated intermediates, which have an anticancer activity (30,31). The cell lines that we used in this investigation may have a low capacity to generate the selenium intermediates, thus the doses we used for the treatment of the cells could perhaps have been substantially higher than that required for an *in vivo* study. Therefore, the proper doses for an *in vivo* study have to be determined.

In addition to SeMet, which is the selenium compound used in the SELECT (selenium and vitamin E chemoprevention trial), other selenium compounds should be evaluated for their effectiveness in similar approaches.

The determination of the proper doses of effective selenium compounds combined with the information regarding cellular and molecular biomarkers obtained from SELECT, should lead to improvements in tumor control by the development of selenium-based multitarget approaches to radiation therapy.

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