Cell Cycle Arrest Biomarkers in Human Lung Cancer Cells After Treatment with Selenium in Culture

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Abstract

In the planning of future intervention trials using chemopreventive agents against lung cancer, it is critical to evaluate the effect on biomarkers implicated specifically in lung carcinogenesis. With the use of the H520 and H522 human lung cancer cell lines, the present study showed that treatment with selenium (in the form of methylseleninic acid) inhibited cell growth, arrested cell cycle progression at G1, and induced apoptosis as a late event. Because H520 cells were more sensitive to selenium than H522 cells (IC50 of MSA was 2.5 or 10 μM for H520 or H522 cells, respectively, at 24 h), a panel of nine cell cycle regulatory proteins known to be involved in G1 → S transition was assessed by Western analysis using whole cell lysate from H520 cells. These nine proteins (DP1, cdc25A, cyclin A, cyclin B1, cyclin D1, cdk1, cdk5, p21WAF1, and GADD153) have been reported previously by our laboratory to be modulated by MSA in human breast and prostate cancer cells. Our data showed that only four (DP1, cdc25A, p21WAF1, and GADD153) of nine biomarkers produced the expected changes after treatment of lung cancer cells with MSA. This finding raises the possibility that the molecular targets sensitive to selenium modulation may be tissue specific. Thus, the selection of selenium biomarkers for evaluation in an intervention trial must be based on empirical data derived from the cancer cell type of interest.

Introduction

Prevention of lung cancer remains an important public health goal because of the large number of people at risk and that current treatment options do not offer promising prognosis for most patients (1). Cigarette smoking cessation leads to only limited risk reduction given that about one-half of newly diagnosed lung cancer patients are former smokers (2). Despite numerous well-designed clinical trials, the search continues for an effective chemopreventative agent against lung cancer (1).

There has been substantial interest in the anticancer activity of selenium-containing compounds. The ongoing Selenium and Vitamin E Chemoprevention Trial is a randomized cohort study designed to evaluate prostate cancer intervention by either agent alone or in combination in >32,400 healthy men (3). Previously, Clark et al. (4) reported a ~50% reduction in the risk of lung cancer by selenium supplementation as a secondary end point in the landmark placebo-controlled NPC trial involving 1312 participants. A recent updated analysis of additional follow-up years resulted in an attenuated risk estimate for the full population in the NPC trial, but there was still a significant 42% decrease in risk among individuals with a low baseline plasma selenium (5). Currently, under the auspices of Eastern Cooperative Oncology Group, there is a Phase III randomized trial to determine the efficacy of selenium in preventing the occurrence of second primary lung cancer in patients with resected stage I non-small lung cancer. Information on this study can be found online (2).

The use of cancer morbidity as an end point in prevention trial is both a costly and long-term endeavor. Therefore, to hasten the discovery process, the evaluation of a chemopreventive agent often necessitates the identification of responsive biomarkers as potential surrogate end points that might predict reduced cancer risk. Proliferative biomarkers have been proposed for analysis in lung cancer chemoprevention trials (6). In the present study, we examined the changes in expression of nine cell cycle regulatory proteins in selenium-treated human lung cancer cells. These nine proteins have been shown previously by our laboratory to be modulated in human premalignant breast cells and human prostate cancer cells exposed to selenium (7, 8). As in the earlier studies, human lung cancer cells were treated with MSA. This is a form of selenium developed specifically for in vitro experiments because cultured cells are sensitive to physiological concentrations of MSA in the medium (9). In addition to the analysis of biomarkers, cell cycle distribution and apoptosis assays were also carried out in lung cells to assess benchmark cellular responses to MSA.

Materials and Methods

Selenium Reagent and Cell Lines. MSA was prepared as described previously (9). The human lung cancer NCI-H522 (adenocarcinoma) and NCI-H520 (squamous) cell lines were

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1 The abbreviations used are: NPC, Nutritional Prevention of Cancer; GADD, growth arrest and DNA damage; MSA, methylseleninic acid; MTT, 3-(4,5-diethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; cdk, cyclin-dependent kinase; PL, propidium iodide; NSCLC, non-small cell lung cancer.

5% CO₂ in a 37°C humidified incubator. For the assays, 2.5 μM MSA. Cultured cells were maintained in an atmosphere of 10% bovine serum, supplemented with penicillin, streptomycin, and gentamicin. Cultured cells were maintained in an atmosphere of 5% CO₂ in a 37°C humidified incubator. For the assays described below, four replicate experiments were carried out per time point and MSA dose.

**Cell Count Assay.** The MTT assay measures viable cells based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolically active cells (10). Cells were seeded in 24-well plates at a density to reach 70–80% confluency at the time of assay. At 48 h after seeding, cells were treated with 2.5, 5, 10, or 20 μM MSA in triplicate. At 6, 12, 18, 24, or 48 h after MSA exposure, 200 μl of MTT was added to each well, and the plates were incubated for 4 h at 37°C. The MTT crystals were solubilized in isopropanol, and the supernatant was transferred to a 96-well plate, and light absorbance was measured at 570 nm using a Spectra Microplate reader (SLT, Salzburg, Austria).

**Cell Cycle Analysis.** Cells were plated in T75 flasks at an appropriate density to reach 70–80% confluency after 48 h in culture. Synchronization of cells was achieved by starving cells in medium with 1% fetal bovine serum for 48 h (cells lost viability in medium completely free of serum). Upon return to culture, cells were treated with 2.5, 5, 10, or 20 μM MSA. The treatment period was for 6, 12, 18, or 24 h. As described previously (7, 8), the posttreatment cells were trypsinized, washed, and fixed in 70% ethanol. The ethanol solution was removed, and cells were stained with PI and analyzed for DNA content using a FACScan Cytometer (Becton Dickinson, San Diego, CA).

**Apoptosis.** Cells were plated as described above. After 48 h, H520 cells were exposed to 2.5 μM MSA for 12, 24, 36, or 48 h, whereas H522 cells were exposed to 5 μM MSA for 48 or 72 h. Adherent cells harvested by mild trypsinization were pooled together with detached cells and stained with biontin-conjugated annexin V, FITC-conjugated streptavidin and PI using the Annexin V-Biotin Apoptosis Detection Kit (Oncogene Research Products, Boston, MA) per manufacturer’s protocol. This assay identifies subpopulations of cells at early or late stage of apoptosis. PI is a DNA binding dye, whereas annexin V has a strong affinity for phosphatidylserine, which is externalized to the cell surface when cells undergo apoptosis. Hence, intact cells would be negative for both annexin V and PI. Cells in early apoptosis would be positive for annexin V and negative for PI. Late apoptotic or necrotic cells are double positive as a result of loss of membrane integrity. Cells were counted by flow cytometry, and the data analyzed with WinList software (Variety Software House, Topsham, ME).

**Western Blot Analysis.** Whole cell lysates were used in Western blot analysis, which was performed as described previously (7, 8). Individual protein bands were visualized by an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoreactive bands were quantified by volume densitometry using the ImageQuant Software (Molecular Dynamics, Sunnyvale, CA) and normalized to actin. The following monoclonal antibodies were used: anti-cdk1/cdc2 (BD Transduction Laboratory, San Jose, CA); anti-cyclin A, cdc25A, cdk5, and p21WAF1 (NeoMarkers, Fremont, CA); and anti-DPI (Oncogene Research, Boston, MA); anti-cyclin B₁ and anti-cyclin D₁ (Santa Cruz Biotechnology, Santa Cruz, CA); and anti-cyclin A, cdc25A, cdk5, and p21WAF1 (NeoMarkers, Fremont, CA). A polyclonal antibody to GADD153 was purchased from Santa Cruz Biotechnology.

### Results

**Effect of MSA on Cell Number.** Both the H520 and H522 cells responded to MSA inhibition of growth in a dose- and time-dependent manner (data not shown). Our laboratory has published extensive and similar results of this nature with respect to human breast and prostate cells (7, 8). To minimize redundancy, we will only point out one salient feature of the MSA effect on the two lung cancer cell lines. The H520 cells were decidedly more sensitive to MSA than the H522 cells. For example, at 12 h of exposure, the IC₅₀ was 5 μM for the H520 cells but was >20 μM for the H522 cells. At 24 h, the IC₅₀ was 2.5 and 10 μM for the H520 and H522 cells, respectively.

**Cell Cycle Block at G₁ by MSA.** The DNA content of PI-labeled synchronized cells was measured by flow cytometry to determine whether inhibition of cell growth was related to cell cycle arrest. In the first experiment, H520 cells were treated with 2.5 μM MSA for 6, 12, 18, or 24 h. As seen in Table 1, MSA treatment resulted in a significant block at G₁-G₂ phase of cell cycle. This was consistent with the MTT assay where MSA caused a marked reduction in cell viability at these time points. The distribution of synchronized cell populations after MSA treatment is shown in Table 1.
the cell cycle at each time point. The block was especially pronounced at 18 and 24 h. Correspondingly, a higher proportion of H520-untreated cells progressed to S phase compared with MSA-treated cells, particularly at the later time points. With the H522 cells, the cultures were treated with 5 \( \mu \text{M} \) MSA because of the reduced responsiveness to MSA as stated above. The cell cycle distribution of treated and untreated H522 cells was not statistically different after 6 and 12 h (Table 1). However, the treated cells exhibited a significant G0-G1 block at the 18 and 24 h time points. For a direct comparison to H520 cells under a similar experimental condition, H522 cells were also exposed to a lower level of 2.5 \( \mu \text{M} \) MSA for 6, 12, 18, and 24 h. No cell cycle arrest at G0-G1 was apparent with this protocol (data not shown).

**Induction of Apoptosis by MSA.** Cells were labeled simultaneously with annexin V and PI and evaluated by flow cytometry to determine whether MSA might cause programmed cell death. Intact cells are negative for both annexin V and PI, early apoptotic cells are positive for annexin V only, and late apoptotic or necrotic cells are characterized by positive staining to both annexin V and PI. As this assay cannot distinguish between necrotic and late apoptotic cells, the percentage of cells positive for annexin V only is considered as a true measure of apoptosis. Thus, we are only presenting the results of annexin V-positive cells. The data in Fig. 1 show that H520 cells exhibited evidence of a steady increase of apoptosis when exposed to 2.5 \( \mu \text{M} \) MSA after 24, 36, or 48 h. However, only the increase at the 48-h time point was statistically significant (\( P < 0.002 \)). There was a suggestion that H522 cells treated with 5 \( \mu \text{M} \) MSA were also sensitive to apoptosis induction after a longer period of exposure (48 and 72 h), but these differences were not significant. Because of the variability of the data, the response was not so clear-cut. Nonetheless, it was apparent that more cells did undergo apoptosis after cell cycle block at G1 upon treatment with MSA.

**Changes in Expression of Cell Cycle Regulatory Proteins by MSA.** An underlying goal of this study was to identify candidate biomarkers for use as intermediate endpoints in future selenium chemoprevention trials of lung cancer. As described above, MSA caused cell cycle block of H520 and H522 cells at G1 transition to S phase. A panel of cell cycle regulatory proteins, which are known to be involved in G1-S transition, was assessed by Western analysis. These proteins included DP1, cdc25A, cyclin A, cyclin B1, cyclin D1, cdk1, cdk5, p21WAF1, and GADD153. We reported previously that these proteins were modulated by MSA in human breast and prostate cancer cells (7, 8). Thus, we wanted to find out if similar effects would be observed in human lung cancer cells. Western blot analysis was carried out in H520 cells because they were more sensitive to MSA than H522 cells. The results are shown in Fig. 2 and Table 2. DP1 and cdc25A were depressed either across all time points or at least in three of four time points. In general, cyclin A, cyclin B1, cyclin D1, cdk1, and cdk5 all showed either very marginal changes or did not exhibit a consistent pattern of change as a function of time (e.g., cyclin D1). In contrast, p21WAF1 and GADD153 were significantly up-regulated by MSA. The near 8-fold increase of GADD153 at the 6-h time point was particularly pronounced.

**Discussion**

The two major reasons for selecting the nine selenium-responsive biomarkers for evaluation in human lung cells are: (a) they represent cell cycle regulatory proteins, which are critical for G1-S transition, and selenium causes cell cycle arrest at G1 in the lung cells; and (b) these same nine regulatory proteins were found to be modulated by selenium in human breast and prostate cancer cells (7, 8). Our data showed that only four of nine biomarkers produced the expected changes after treatment of...
lung cancer cells with selenium. This finding raises the possibility that the molecular targets sensitive to selenium modulation may be tissue specific and that the information obtained from one cancer cell type may not be applicable to another cancer type. To a certain extent, this revelation is not entirely surprising. We already know that selenium affects a multitude of targets (7, 8). It would be rather unusual if all cell types respond in an identical manner to selenium at the molecular level. Therefore, the selection of selenium biomarkers for evaluation in an intervention trial must be based on empirical data derived from the cancer cell type of interest. Extrapolating the information from other cell types is likely to produce misleading conclusions.

Our data in lung cells showed that selenium treatment down-regulates the expression of DP1, which is a dimerization partner of E2F proteins. Because E2Fs are known to positively regulate the transcription of cdc25A, a decrease in the formation of E2F and DP1 dimers would lead to a depressed level of cdc25A (11), which is consistent with our finding. The reduced phosphatase activity of cdc25A increases the ratio of inactive to active cdk2, thus interfering with cell cycle progression from G1 → S.

GADD153, a member of the GADD-inducible gene family, is the most highly modulated gene at the early time point in lung cells treated with MSA. An early response gene like GADD153 suggests that it may be a key trigger of selenium action. Although GADD153 is known to increase in response to a variety of growth arrest and DNA damage signals (12–14), it should be noted that the mechanism of GADD153 induction by selenium is unique because MSA does not cause single strand DNA breaks (9). In addition to lung cells, the induction of GADD153 by MSA is present in every cell line we have examined, including two human breast cancer cells (7) and four human prostate cancer cell lines (Ref. 8 and unpublished data).

The overexpression of GADD153 has been known to lead to cell cycle block and apoptosis (15–17); these effects are consistent with that of selenium. Little information is currently available on the specific role of GADD153 in lung cancer development. Interestingly, a recent study by Satoh et al. (18) demonstrated that the level of GADD153 protein was increased in non-small cell lung cancer cells after exposure to troglitazone, an antidiabetic drug that has been associated with growth inhibition of a variety of malignancies. These investigators also concluded that GADD153 might be a key candidate gene in growth suppression and apoptosis induction upon the examination of 160 related genes by array analysis.

The clinical significance of p21WAF1 in NSCLC is gradually emerging. Recently, Shoji et al. (19) reported that in a study of 233 patients with completely resected pathological stage I to IIIA NSCLC, the 5-year survival rate of p21WAF1-positive patients was significantly higher than that of p21WAF1-negative patients. Using the serial analysis of gene expression technique in nine NSCLC libraries, Nacht et al. (20) found that p21WAF1 was consistently underexpressed in adenocarcinomas and, to a lesser extent, in squamous cell lung cancer also. On the other hand, Komiya et al. (21) noted that the favorable prognosis associated with positive p21WAF1 status was restricted to squamous cell lung cancer only. With respect to the impact of p21WAF1 up-regulation on growth arrest and apoptosis, Yu et al. (22) recently demonstrated a compelling correlation between the changes of these cellular events and the induction of p21WAF1 by the anticancer drug FK228 (an histone deacetylase inhibitor) in a panel of NSCLC cell lines. On the basis of the above evidence, we propose that agents that can induce p21WAF1 expression such as selenium may delay the appearance of lung cancer.

An important next step would be to evaluate MSA in premalignant lung cells, given that most chemoprevention trials include individuals with the greatest risk for developing lung cancer (23) such as former or current smokers with persistent dysplasia (24). Studies are currently underway to evaluate the effect of selenium in cell cultures from bronchial epithelial brushes from high-risk lung cancer patients. Despite the recognized need to do additional research in premalignant lung cells, we are unaware of any human bronchial epithelial cell line derived directly from precursor lesions. Although in vitro molecular studies of precursor lesions of the lung have been conducted with several agents (25–27), the source of tissue has typically been developed in individual laboratories and involved tobacco-carcinogen transformed, virally immortalized normal bronchial cells. Ongoing studies of sputum cytology (28, 29) and laser-induced fluorescence endoscopy detection (30) show promise for acquiring neoplastic bronchial tissue for future studies.

The main goal of this article was to evaluate molecular mechanisms of action of selenium in a lung cancer model that might explain the in vivo effects seen in the NPC trial, with the ultimate purpose of identifying candidate biomarkers for use as surrogate end points in a future trial of high-risk individuals. Optimally, molecular targets for use in chemoprevention studies should be validated in both preneoplastic and malignant cells, yet it has been suggested that in vitro data gathered from advanced disease can be relevant to primary prevention given that pharmacological interventions could also serve to arrest progression in occult stages (24). Of note, the nine biomarkers selected for our current study were found to be selenium responsive in a previous in vitro study of premalignant tissue of the breast (7). Our findings that only four biomarkers showed the expected responses to selenium reaffirm the urgency to identify additional lung-specific markers for the effect of selenium.

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References

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