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Research Article

Selenoprotein deficiency enhances radiation-induced micronuclei formation

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The availability of selenium and the levels of specific selenoproteins might affect cancer risk by influencing the ability of DNA damaging agents to cause genomic instability and mutations. Transgenic mice that express reduced levels of selenoproteins and previously shown to be more susceptible to pathology associated with cancer development were used to study this possibility. These mice were exposed to X-rays and DNA damage assessed in the erythrocytes, where micronuclei formation was higher compared to the same cells obtained from irradiated wild-type controls. To determine whether the selenoprotein glutathione peroxidase-1 (GPx-1) might be involved in this protection, its levels were reduced by siRNA targeting in LNCaP human prostate cells. UV-induced micronuclei frequency was higher in these cells compared to control-transfected cells. These results indicate a role for selenoproteins in protecting DNA from damage and support human data implicating GPx-1 as a possible target of the chemoprotective effect of selenium.

Keywords: DNA damage / Glutathione peroxidase / Selenium / Selenoproteins

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1 Introduction

Selenoproteins represent a class of peptides distinguished by the presence of the amino acid selenocysteine (Sec). Sec is incorporated cotranslationally in response to UGA codons designated as the Sec codon by the SECIS identity element located within the coding sequence in prokaryotes or in the 3′-untranslated region in eukaryotes [1–3]. The human genome contains 25 selenoprotein genes, often containing Sec at the enzyme's active site, and several of these have been shown to have anti-oxidant functions [4, 5]. Selenoproteins have received considerable interest as the possible mediators of the anticancer effects observed when animals or humans have their diets supplemented with low, nontoxic levels of selenium, although definitive proof of this particular role for selenoproteins has not yet been provided. Human genetic data implicating a subset of proteins in the selenoprotein family in cancer risk and etiology have been reviewed [6].

A useful tool to examine the role of selenoproteins in biological processes was developed by engineering transgenic mice, referred to as i6A$^-$, that express reduced selenoprotein levels due to the introduction of dominantly acting mutant Sec tRNA [7]. Although most selenoproteins are present at reduced levels and in most organs in this animal, it is otherwise without phenotype. A role for selenoproteins in cancer prevention was indicated by data obtained using this mouse model. When i6A$^-$ were bred against C3(1)Tag mice that develop prostate cancer due to the directed expression of the SV40 oncogene in that organ, the resulting bigenic offspring exhibited accelerated prostate pathology associated with cancer development as compared to that typically observed in the C3(1)Tag mouse [8]. Consistent with these data, it was also reported that i6A$^-$ mice were more susceptible to azoxymethane-induced aberrant crypt foci in their colons, a generally accepted preneoplastic marker for colon cancer development, as compared to wild-type (WT) controls [9]. Data presented in this study indicated a role for both selenoproteins and low M_r selenocompounds in the observed reduction of the colonic preneoplastic lesions.

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Abbreviations: GPx-1, glutathione peroxidase-1; **Sec**, selenocysteine; **WT**, wild type

The mechanisms by which selenium and/or selenoproteins can reduce cancer risk remain to be determined. Increased cell death of preneoplastic lesions, enhancement of immune function, and protection from DNA damage are all possible explanations. In this study, we report continued efforts using the i6A$^-$ mouse model and evidence that one particular selenoprotein implicated in cancer risk by *in vitro* and *in vivo* data is a candidate beneficial enzyme.

2 Materials and methods

2.1 Animals

All animals were handled in accordance with the principles and procedures of the *Guide for the Care and Use of Laboratory Animals* by the Institute of Laboratory Animal Resources, National Academy Press, Washington, DC, 1996 and the experiments were approved by the Institutional Animal Care and Use Committee. WT FVB/N or i6A$^-$ transgenic animals developed in the FVB/N background were kindly provided by Dolph Hatfield at the NIH, and maintained on a standard AIN-93G diet purchased from Harlan Teklad (Madison, WI) until being irradiated with 4 Gy of X-rays or sham irradiated at 6 – 7 months of age using a Cs-137 irradiator (J. L. Sheperd and Associates irradiator Model 143-68) in a well ventilated holding cylinder. Twenty-four hours post-treatment, the animals were sacrificed by asphyxiation with CO_2 and the indicated organs were harvested for analysis. All reported animal studies were the result of six or seven animals *per* group and the error bars in the figure represent the SD of the mean.

2.2 Micronuclei analysis

Quantification of micronuclei was conducted using a modification of the procedure described by Fenech and Morely [10]. Femurs were dissected and the bone marrow was flushed into DMEM. The suspension was centrifuged, several drops of fetal calf serum (FCS) were added, and the pellet was mixed thoroughly. Smears were drawn onto pre-cleaned-coded slides using a drop of the resultant suspension. The slides were air dried, fixed in absolute ethanol, stained with 0.1% ethidium bromide in Sorensen's buffer (pH 6.8), and washed twice in Sorensen's buffer. The appearance of micronuclei was determined by observation with a fluorescence microscope using a 40 × objective. A minimum of 500 – 1000 erythrocytes were evaluated by an investigator who was blinded to the source of the cells. Quantification of GPx enzyme activity by coupled spectrophotometric assay was as previously described [8].

2.3 Cell culture studies

In order to reduce glutathione peroxidase-1 (GPx-1) levels in LNCaP cells, an siRNA targeting construct was generated using the mRNA target sequence AAGAACGAAGAGATTCTGAAT spanning positions 342 – 362. Using this target sequence, a hairpin siRNA template oligonucleotide was synthesized and ligated into pSilencer 2.1-U6 siRNA vector (Ambion, Austin, TX). The empty pSilencer vector without insert and the siRNA targeting construct were transfected by electroporation into LNCaP cells using Amaxa Biosystem's Nucleofector I. Transfected cells were selected with hygromycin B (2 mg/mL), expanded, and screened for GPx enzyme activity. Micronuclei were quantified after exposure to UV. For UV exposure, cells in logarithmic growth were irradiated with UV (254 nm) at room temperature using an UV-crosslinker (Stratalinker 2400, La Jolla, CA) at 12 J/m^2. For irradiation, media was removed, the cells washed with sterile cold PBS and then exposed to UV for under 6 s in PBS. After treatment, cultures were incubated with medium containing cytochalasin-B (3 μg/mL) for 48 h postirradiation. After the stipulated time, medium containing cytochalasin-B was removed and the cells were washed with PBS, collected by trypsinization, centrifuged, and subjected to mild hypotonic treatment (0.57% ammonium oxalate) for 6 min at 37°C. The cells were centrifuged and the resultant cell pellet was fixed overnight in 70% ethanol, at which time the cells were washed three times with Carnoys fixative (3:1 methanol/acetic acid), resuspended in a small volume of Carnoy's fixative and spread onto coded slides, stained with 10% v/v Giemsa for 30 min, washed, and dried. A minimum of 1000 binucleate cells (BNCs) with well-preserved cytoplasm were scored for the presence of MN by a blinded observer to avoid bias. Experiments were repeated at least three times and the results were analyzed by one-way analysis of variance (ANOVA).

3 Results

3.1 Erythrocytes from selenoprotein deficient mice display enhanced sensitivity to DNA damage

The i6A$^-$ mouse has previously been shown to be more susceptible to cancer of the prostate and colon compared to WT animals [8, 9]. One possible way this might occur is if the immune system of that animal was somehow compromised: selenium has been shown to have effects on immune function [11] and it is therefore possible that the reduction in selenoprotein levels in these mice would have a similar effect. However, the B- and T-cell profiles of the i6A$^-$ mice were indistinguishable from WT control mice (data not shown). Other mechanisms of increased cancer susceptibility were therefore investigated.

In order to determine whether cellular DNA was more susceptible to damage in the i6A$^-$ genetic background, it was investigated whether erythrocytes obtained from femurs of i6A$^-$ mice were more susceptible to DNA dam-

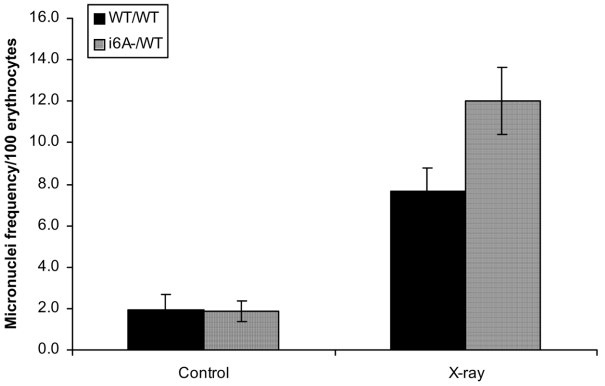

Figure 1. Micronuclei frequency in the erythrocytes of i6A$^-$ and WT mice following irradiation. Selenoprotein deficient and WT mice were exposed to 4 Gy of X-ray, erythrocytes were harvested from the femurs and microscopically examined for the appearance of micronuclei. Baseline micronuclei frequency in the erythrocytes was not significantly different between the untreated i6A$^-$ and WT mice, however X-ray induced micronuclei frequency in the i6A$^-$ mice was significantly higher compared to the WT mice ($p < 0.001$). The studies in the figure involved six and seven animals *per* group and the error bars represent the SD of the mean.

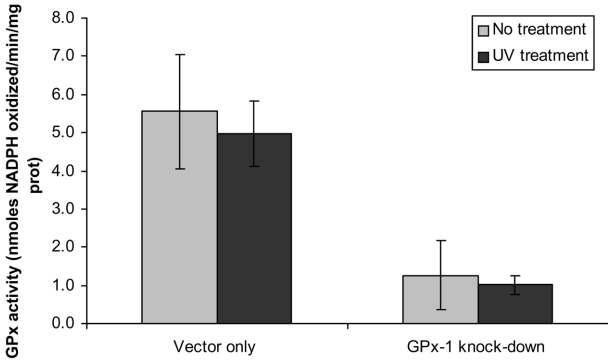

Figure 2. GPx activity in untreated and UV treated vector-only and GPx-1 knockdown transfectants. Mean activities from three independent assays are expressed as nanomoles NADPH oxidized/min/mg protein ± SD. LNCaP were transfected with a GPx-1 targeting siRNA construct or vector and total GPx activity was determined. GPx activity was significantly lower in the GPx-1 knockdown cells compared to vector-only controls ($p < 0.001$) in untreated and UV treated groups.

age, as measured by the formation of micronuclei following exposure to ionizing radiation. Both i6A$^-$ and WT mice were exposed to 4 Gy of X-rays and erythrocytes were harvested and microscopically examined for the appearance of micronuclei. This assay is commonly used, which permits the indirect evaluation of DNA damage susceptibility by measuring the appearance of a secondary nucleus (micronucleus) formed in a dividing cell [12, 13]. The data shown in Fig. 1 indicate that the baseline levels of micronuclei observed in both i6A$^-$ and WT mice were not significantly different, but the relative number of cells with both single and double micronuclei was significantly higher when erythrocytes obtained from irradiated i6A$^-$ mice were compared to WT controls.

3.2 Knockdown of GPx-1 enhances the sensitivity of human prostate cells to DNA damage

The i6A$^-$ mouse is deficient in several of the 24 mouse selenoproteins, and whether the reduction of any one of these results in the increased susceptibility to micronuclei formation reported above is an important issue. Given the previous data on increased prostate carcinogenesis in the i6A$^-$ background [8], and human genetic data implicating one selenoprotein, GPx-1, in cancer risk and development [6], it was decided to reduce GPx-1 levels in human prostate cells using siRNA and determine whether there was a consequential effect on micronuclei formation. Previously, we have shown that human breast cancer cells engineered to over-express GPx-1 were less susceptible to UV-induced micronuclei formation [14]. We therefore reduced GPx-1 activity by stably transfecting human LNCaP prostate cells with a GPx-1 targeting siRNA construct and compared UV-induced micronuclei formation to the same cell line transfected with only the targeting vector. Transfectants were selected and GPx activity was determined by a direct enzyme assay, establishing the efficacy of the siRNA construct (Fig. 2). As shown in the figure, the siRNA transfectant had reduced baseline GPx activity. UV treatment did not affect GPx activity in either of the transfectants. siRNA and vector-only transfectants were exposed to UV and cells with discernable micronuclei were enumerated. These results are presented in Fig. 3 and indicate that the levels of micronuclei observed between the cells differing in GPx activity did not change in unirradiated cells, and that both cell types showed a significant increase in the micronuclei frequency when exposed to 12 J/m^2 of UV. Noticeably, the micronuclei frequency was significantly higher in cells with lower GPx activity.

4 Discussion

Both human and animal data have supported the chemopreventive potential of selenium supplementation, and there has been significant focus on the selenoproteins as a possible mediator of this element's benefits. The development of the i6A$^-$ mouse model offers an ideal opportunity to investigate the role of selenoproteins in biological processes independent of secondary effects that might accompany changes in selenoprotein levels when dietary selenium intake is altered. Selenoproteins are essential for development, as evidenced by the early developmental lethality reported for the Sec tRNA knockout mouse [15, 16], and the reduction in selenoprotein levels achieved in the i6A$^-$

Micronuclei frequency/100 cells		
	Untreated	**UV**
Vector only	1.5 ± 0.19	2.6 ± 0.34
GPx-1 knock-down	1.3 ± 0.36	3.0 ± 0.19

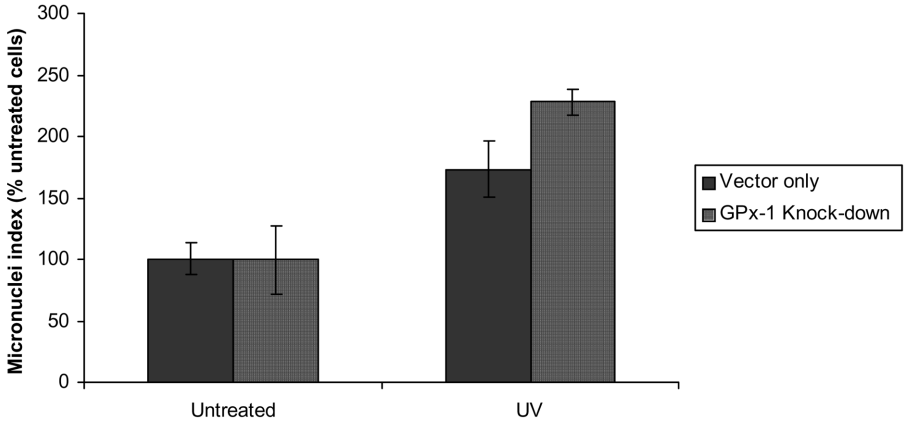

Figure 3. Micronuclei induction in GPx-1 knockdown and vector-only transfectants following UV irradiation. Inset table shows micronuclei frequency *per* 100 cells. The data presented are the result of three independent experiments with each data point obtained in triplicate. Values are expressed as the mean ± SD. Micronuclei frequency was not significantly different between the untreated control and GPx-1 knockdown transfectants (inset table) but was significantly increased postirradiation ($p < 0.001$). UV-induced micronuclei frequency was significantly higher in the GPx-1 knockdown cells compared to vector-only controls ($p < 0.05$). Within and between group comparisons were made by two-sided Student's *t*-test using the statistical package SigmaStat version 2.03 (SPSS, Chicago, IL). A value of $p < 0.05$ was considered significant.

mouse is likely to yield information more biologically relevant than one might obtain using animals engineered to be null in one or more selenoproteins. Two reports in the literature indicate that the reduction in selenoproteins achieved in this mouse model resulted in an increased incidence of preneoplastic lesions in both the colon and prostate [8, 9], indicating the utility of the i6A$^-$ mouse for studies of chemoprevention. The studies presented herein represent early steps to elucidate the mechanism by which selenoproteins can influence cancer risk.

Initial studies to determine whether the immune system was significantly compromised in the i6A$^-$ mouse failed to find any significant differences between the B- and T-cell population in the spleen of these mice compared to WT animals. Subsequent studies examined whether there was increased sensitivity to DNA damage in this genetic background. It was previously shown that adding low levels of selenium, 30 nM in the form of sodium selenite, to the media of CHO cells caused a reduction in X-ray induced mutations and a four- to five-fold increase in GPx activity [17]. In addition, both selenium supplementation and increased expression of GPx-1 were sufficient to reduce the levels of micronuclei observed post-irradiation of human MCF-7 breast carcinoma cells [14]. The studies presented here demonstrating increased micronuclei frequency following irradiation are consistent with the MCF-7 results and extend the observations from cultured cells to animals. The observation of increased DNA damage in GPx-1 reduced prostate cancer cells also suggests that this protective effect is a general phenomenon that is not cell-type specific.

How GPx-1 levels might influence DNA damage remains an area of future investigation. Given the data indicating that GPx-1 levels influence DNA damage induced by two different qualities of radiation, X-rays and UV, one possible mechanism of action would be the modulation of signaling pathways that ultimately regulate DNA damage repair. Overexpression of GPx-1 can protect cells from UV-induced micronuclei formation [14] and stimulate the expression of GADD45 in MCF-7 cells [18], a protein likely to play a significant role in the response to DNA damage [19, 20]. Selenium supplementation can also protect against UV-induced micronuclei formation in MCF-7 cells, but not in rodent fibroblasts that are null for BRCA1, a gene mutated in breast cancers, implicated in DNA repair, and an activator of GADD45 [14]. These observations offer the possibility that the benefits of selenium might be mediated through its stimulation of GPx-1 and the subsequent enhancement of the repair of DNA damage, perhaps involving the BRCA1/GADD45 pathway.

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The authors have declared no conflict of interest.

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