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Selenium Compounds Regulate p53 by Common and Distinctive Mechanisms

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Abstract. *Selenium compounds show much promise in the prevention of prostate and other human cancers. Various selenium chemical forms have been shown to differ widely in their anticancer properties. The main dietary form is selenomethionine, which we showed modulated p53 activity by causing redox regulation of key p53 cysteine residues. In the current study we included other selenium chemical forms, sodium selenite and methyl-seleninic acid. All three forms are relevant selenium sources in human populations. All three forms can affect p53 activity defined as trans-activation of a p53-dependent reporter gene. In addition to the reduction of cysteine sulfhydryl groups, p53 phosphorylation was also affected in cells treated with selenium compounds. Methyl-seleninic acid caused phosphorylation of one or more p53 threonine residues, but did not affect any known serine phosphorylation sites. By contrast sodium selenite caused phosphorylation of p53 serines 20, 37 and 46 known to mediate apoptosis. Selenomethionine did not cause detectable phosphorylation of p53 serines or threonines. Our data show that, although p53 modulation may be a common denominator of selenium compounds, specific mechanisms of p53 activation differ among selenium chemical forms. Post-translational modifications of p53 are determinants of p53 activity and probably affect the threshold for p53-mediated functions. Different selenium chemical forms may differentially modify p53 for DNA repair or apoptosis in conjunction with a given level of endogenous or exogenous DNA damage.*

Abbreviations: XP, *xeroderma pigmentosum*; SeMet, seleno-methionine; MSA, methyl-seleninic acid; NaSel, sodium selenite; APE/Ref1, bifunctional protein encoding apurinic endonuclease and redox factor-1

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Selenium compounds, in various chemical forms, have a twenty-year history of cancer prevention in rodent models of mammary and colon carcinogenesis (1, 2). The chemical forms exhibiting most efficacy in cancer prevention are typically organic compounds that are relatively non-toxic, the prototype form being selenomethionine. Recently, other organoselenium compounds including seleno-methylselenocysteine and 1,4-phenylbis(methylene)selenocyanate (3) have demonstrated superior cancer prevention activity compared to selenomethionine. Selenium is used in phase II-III clinical trials for prostate cancer prevention, and there is evidence for prevention of other human cancers as well (4). Originally, mixed naturally-occurring selenium compounds were administered in the form of a yeast extract (5), although selenomethionine is the major constituent of dietary selenium and is being administered in current trials (6). A notable dietary source of selenium is Brazil nuts which have a high selenomethionine content (7). Logically, there is much interest in selenium and reasonably there is need to ascertain the molecular basis of selenium action. Although it is likely that the effects of selenium are pleiotropic (8), one important tumor suppressor protein, p53 has been shown to be selenium responsive. In particular p53 cysteine residues 275 and/or 277 are responsive to supranutritional levels of selenomethionine supplied in the culture medium (9, 10). It is established that p53 is of paramount importance in carcinogenesis, underscoring its potential importance as a selenium target. Indeed, given that approximately 70% of all human cancers exhibit defective p53, it is to date the most ubiquitous gene defect in human carcinogenesis. Mice carrying gene disruptions of p53 are cancer-prone, although the latency period for development of lymphomas is short so that analysis of other cancers *e.g.* mammary cancers and effects of chemoprevention in these mice has been challenging (11).

Moreover, selenomethionine (SeMet) and other seleno-amino acids, in addition to utilization as selenocysteine-containing selenoproteins *e.g.* thioredoxin reductase, are also converted to low-molecular-weight metabolites (12). For example, a fraction of the input selenomethionine is converted to methylselenol by an L-methionine-α-deamino-

γ-mercaptomethane lyase enzyme (13), which may be rate-limiting in some cell lines and tissues. The availability of selenium in a form that does not require conversion to methylselenol, allows for tissue culture experiments to address the role of metabolities apart from seleno-amino acids (14). One potentially active form is methyl-seleninic acid (MSA) which we and others have incorporated into cell culture-based studies. By contrast, genotoxic selenium compounds exemplified by sodium selenite (NaSel) have been widely used as a selenium source but additionally cause DNA damage in the form of strand breaks akin to ionizing radiation. More studies are needed to integrate the various selenium forms in their activation or modulation of molecular targets. The physiological range for selenium is 1-5 μM, although extrapolation to the human population is complex owing to various naturally-occurring chemical forms that differ in relation to geography.

P53 is a transcription factor that regulates as many as 100 downstream effector genes whose products are involved in cellular responses to DNA damage. In addition to obvious roles in apoptosis and cell cycle checkpoint control, p53 regulates genes *e.g.* XPC (the product of the *xeroderma pigmentosum* type C gene) involved directly in DNA repair (15). Additional components of the DNA repair branch of the p53 pathway are p48XPE (the p48 subunit of the *xeroderma pigmentosum* type E gene) and Gadd45a (growth arrest and DNA damage) genes. Mice carrying disruptions of Gadd45a are cancer-prone (16) and exhibit defective DNA repair (16, 17), similar to human patients carrying inborn defects in XPC or XPE genes (18).

In this paper, we examined three prototype forms of selenium: Non-genotoxic compounds SeMet (9, 10) and MSA (14), and the genotoxic compound NaSel, toward targeting p53. All three compounds promoted reduction of p53 cysteine residues 275 and 277, and all three enhanced p53 activity toward a p53-responsive gene promoter element. MSA and NaSel affected p53 at lower concentrations compared to SeMet. Besides cysteines 275 and 277 we examined additional post-translational modifications on p53 protein that may be altered in response to selenium. Specifically, NaSel promoted the phosphorylation of p53 serine residues, while neither SeMet nor MSA had any demonstrable effect on serine phosphorylation. MSA did however cause phosphorylation of one or more p53 threonine residues. Because SeMet and MSA are non-genotoxic (14) *i.e.* they mediate cellular responses *via* signal transduction pathways rather than by DNA damage, phosphorylation of serines or threonines may distinguish between modes of signaling to p53. These data suggested that different selenium compounds may selectively modify p53 for DNA repair or apoptosis. One implication is that by affecting the setpoint for p53 activity, serum selenium concentrations may modulate cellular responses to DNA-damaging agents.

Materials and Methods

Cell lines and treatments. H1299 human lung cancer cells were used in transient co-transfection experiments because they carry endogenous deletions of both p53 alleles (9). Cells were cultured in RPMI1640 plus 10% fetal bovine serum. Baseline selenium in the medium is about 100 nM, and was augmented with additional selenium compounds at indicated concentrations. P53-/- mouse embryo fibroblasts were from a prior study and were obtained from Dr. Michael B. Kastan, St. Jude Childrens Hospital (9). Fibroblasts were cultured in DMEM 4.5g glucose/L plus 10% fetal bovine serum. Cells were treated with selenium compounds at concentrations and durations indicated. Selenomethionine (SeMet) and sodium selenite (NaSel) were from Sigma, St. Louis, MO, USA. Methyl-seleninic acid (MSA) was provided by Dr. Howard Ganther, University of Wisconsin and was prepared as described (12). In addition to H1299 cells, the MCF7 human breast cancer cell line wild-type for p53 was also used to demonstrate p53 activation by selenium compounds and disruption of selenium signaling to p53 by the Ref1 dominant-negative mutant.

P53-dependent reporter assay. Reporter assays were as described previously (9, 10). Plasmid pG13-CAT, which carries 13 repeats of a cognate p53-binding site fused to a minimal gene promoter element, drives expression of the bacterial chloramphenicol acetyltransferase (CAT) gene in the presence of functional p53. The assay is a definitive measure of p53 function (19). H1299 human lung cancer cells null for endogenous p53 genes were transiently transfected with pG13-CAT together with pCMV-wildtype p53 or pCMV-mutant p53 (val-ala codon 143) in a ratio of 5:1 delivered with Fugene reagent (Boehringer-Mannheim, Indianapolis, IN, USA). Plasmid uptake proceeded for 16 hours, then RPMI 1640 plus 10% fetal bovine serum replaced. CAT enzyme activity was determined after 20-24 additional hours of incubation. CAT enzyme was determined by a sensitive ELISA assay (Boehringer-Mannheim). Data were averaged from three or more independent transfection experiments.

Expression plasmids. Vectors encoding wild-type or mutant (val-ala codon 143 allele) p53 were pCMV3 plasmids as described previously (20). APE/Ref1 (referred to for our purposes as simply Ref1) was expressed from the CMV promoter in pcDNA3.1 provided by Dr. Mark R. Kelley, Indiana University (9). The Ref1 C65A mutant carries an alanine substitution at the codon 65 redox center and is inactive in reduction of p53 cysteine 275/277 sulfhydryl groups (9, 21, 22).

Western blots. Immunoblotting was conducted as described previously (9, 10). Antibodies were as follows: for p53, Abs 421 and D01 were used (both from Oncogene Research Products, San Diego, CA, USA), as well as phospho p53-specific Abs (Cell Signaling Inc., Beverly, MA, USA). Phosphothreonine Ab 1E11 was from Oncogene Research Products. D01 Ab coupled to agarose was purchased from Santa Cruz Biotech, CA, USA, and was used to immunopurify p53 from transfected cells for phosphothreonine determination. Rabbit polyclonal Ab H165 to Gadd45a and goat polyclonal Ab to p48XPE were from Santa Cruz. Mouse monoclonal Ab to APE/Ref1 was from Novus Biologicals, Littleton, CO, USA. Monoclonal Ab to PCNA was from Oncogene Research. Primary Abs were incubated with nitrocellulose membranes overnight in 4% nonfat dry milk in PBS. Molecular size markers (Kaleidoscope markers purchased from Bio-Rad Inc., Hercules, CA, USA) were resolved by electrophoresis and

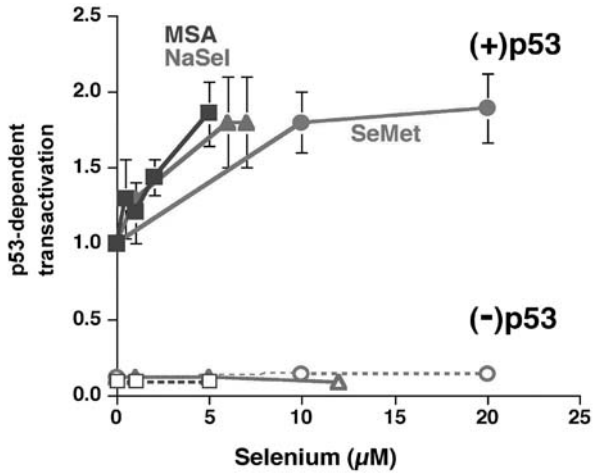

Figure 1. *Transactivation of a p53-dependent reporter gene by selenium compounds. The p53-dependent reporter pG13CAT which carries 13 p53-binding sites and a minimal promoter element driving the chloramphenicol acetyltransferase (CAT) gene, was introduced to a p53-null transfectable cell line H1299 in transient co-transfection experiments together with wild-type p53 (closed symbols) or a mutant-p53 expression vector (open symbols). After 24-h expression, cells were treated with selenium at indicated concentrations and incubated for an additional 16 h, and CAT activity determined. CAT expression was not observed in p53-mutant transfectants (open symbols). Selenium in each of the three forms resulted in enhancement of p53-dependent transactivation of the reporter construct. MSA and NaSel were active at lower concentrations compared to SeMet. A positive control, UV-radiation (20 Jm-2) activated p53 by 2-4 fold in line with Zhan et al. (44) (not shown). The physiological range for selenium is 1-5 μM although extrapolation to the human population is complex owing to various chemical forms in human diets. The y axis represents relative p53 transcriptional activity, normalized to selenium-untreated cells which were also transfected, arbitrarily assigned a value of 1.0. Thus the increase in p53 activity observed in selenium-treated samples was not due to stress of transfection. Error bars represent mean ± S.D. of three independent experiments conducted in quadruplicate.*

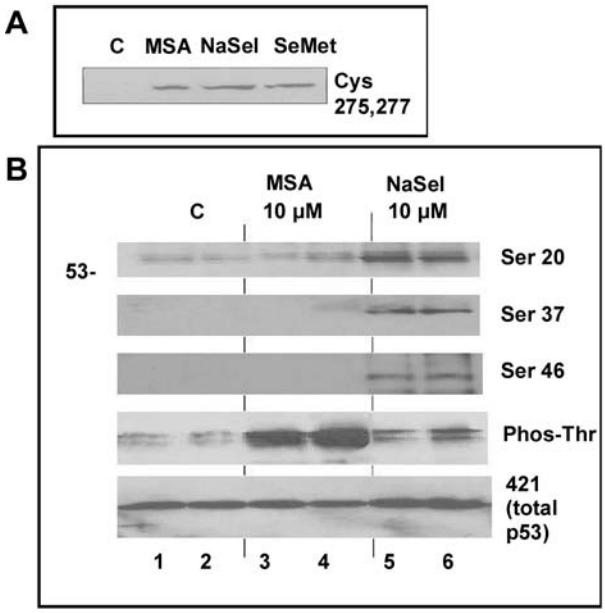

Figure 2. *Post-translational modifications of p53 protein affected by selenium compounds shown by modification-specific antibodies (Western blots). A wild-type p53 expression vector was transfected to a p53-null transfectable cell line H1299 and allowed to express for 48 h. Selenium compounds were added to the medium for an additional 5 h to induce post-translational modifications on p53 protein. A 5-h timepoint was chosen to examine proximal signaling to p53 by selenium without secondary effects i.e. cell death. In 5 h, there was no evidence of cell death (trypan blue staining >95% viability). Because of the short-term endpoint of the experiment, the supra-physiological concentration of 10 μM was used. A) All three selenium forms promoted reduction of cysteines 275/277 sulfhydryl groups (9), but differed in p53 phosphorylation (B). The reduction of p53 cysteine residues was determined by an N-ethylmaleimide conjugate which reacts with free sulfhydryl groups on p53 (9). The N-ethylmaleimide-modified p53 product is shown. B) MSA caused phosphorylation of one or more threonine residues, but did not affect serine phosphorylation. A broad-specificity anti-phosphothreonine Ab 1E11 was used against p53 immune complexes. Two other phosphothreonine Abs, 14B3 and 4D11 (Oncogene Research Products) gave identical results. The phosphorylation patterns of serines versus threonines appears to be a molecular distinction between genotoxic selenium (NaSel) compared to non-genotoxic selenium (MSA). SeMet did not cause detectable phosphorylation of p53 serines or threonines. Total p53 levels derived from the transfected plasmid were not affected, as detected by the Ab421 antibody to total p53. Duplicates are shown; C, control lanes of transfected cells not treated with selenium as a control for endogenous phosphorylation status of specific residues.*

transferred to the membranes for protein size alignment. Second antibodies were horseradish peroxidase conjugates purchased from Sigma. Blots were developed by chemiluminescent substrate (Pierce Chemical, Rockford, IL, USA) and exposed to X-ray film.

The Ab1620 epitope corresponds to transcriptionally-active p53 (23). Immune complexes were collected with Ab1620 (Oncogene Research Products) on protein A/agarose beads. Immunodetection of p53 was by protein A-peroxidase-conjugate (9).

Detection of reduced p53 cysteine residues by reactivity with N-ethylmaleimide was as previously (9). Briefly, an expression vector that encodes a 20 kD fragment of p53 containing only cysteines 275 and 277 was used. The N-ethylmaleimide reaction product was detected by Western blotting of transiently transfected and selenium-treated cells. N-ethylmaleimide-modified p53 fragment exhibited an altered electrophoretic mobility and was thus distinguishable from unmodified fragment.

Host-cell reactivation. Experiments were conducted as described previously (9, 10, 20). The technique utilizes a UV-irradiated CAT reporter plasmid, pSV2-CAT driven by the strong SV40 promoter. UV-treatment introduces up to ten transcription-blocking UV-photoproducts per plasmid molecule. UV-irradiated plasmids exhibit less than 5% of CAT activity compared to undamaged plasmid when transfected to DNA repair defective *xeroderma pigmentosum* XPA cells (20). When introduced to DNA repair-competent cells, the plasmid is reactivated to about 50% of undamaged controls by virtue of removal of transcription-blocking lesions. Thus plasmid reactivation reflects DNA repair capacity of a given cell line and treatment.

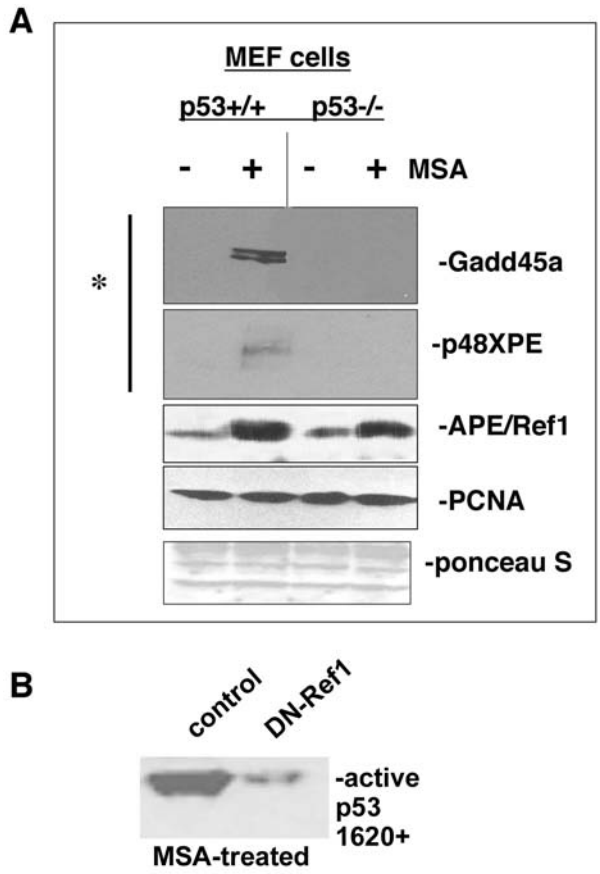

Figure 3. *Regulation of the DNA repair branch of the p53 pathway by selenium compound MSA. A) DNA repair protein expression in p53-wild-type and p53-null mouse fibroblasts treated with 1 μM MSA (Western blots). Consistent with a DNA repair and survival response, DNA repair proteins were elevated in MSA-treated cells 15 h after treatment. Gadd45a and p48XPE genes are p53-regulated (asterisk), while another DNA repair protein apurinic endonuclease (APE/Ref1) is elevated by MSA treatment but its expression is p53-independent. APE/Ref1 is a bifunctional protein that also regulates p53 by its Ref1 redox domain (9). PCNA expression is relatively constant. Ponceau S staining of the membrane indicates equivalent loading of the wells. Basal levels of Gadd45a and p48XPE are not detectable in untreated cells perhaps due to limiting serum selenium concentration in DMEM/10% fetal bovine serum. B) Selenium signaling to p53 is blocked by a dominant-negative Ref1. Cells were co-transfected with wild-type p53 and empty vector (control) or the C65A Ref1 mutant vector. After 24 h, cells were treated with 5 μM MSA for 8 h and the active conformation of p53 was assayed by immunoprecipitation with Ab1620 and immunodetection of p53 (23). No evidence of cell death was observed during the short duration of selenium exposure.*

Results

p53 response to selenium. The implication of p53 as a molecular target of selenium is important because p53 mediates cellular responses fundamental to carcinogenesis including cell cycle arrest, apoptosis and DNA repair. In the case of SeMet, only DNA repair was observed at lower (biologically-relevant) selenium concentrations (9). It is not clear if SeMet is active in amino acid form or if it requires metabolic conversion to lower-molecular weight forms to exert anticancer activities (14). We used MSA as representative of a preformed selenium metabolite. Similar to SeMet, MSA enhanced trans-activation of a p53-dependent reporter gene (Figure 1) and promoted reduction of p53 cysteine 275/277 sulfhydryl groups (Figure 2). Also similar to SeMet (9), MSA did not affect phosphorylation of p53 serine residues (Figure 2). Neither SeMet nor MSA cause DNA damage directly (14) indicating that they regulate p53 by a signal transduction mode independent of DNA damage (9). Physiological serum selenium levels range from 1-5 μM (5).

We measured DNA repair responses to each selenium compound, in line with the evidence that p53 can promote DNA repair (15, 17, 20). MSA (0.5-1 μM) promoted a DNA repair response to UV-radiation (results not shown), similar to our prior work using SeMet (9, 10). The prediction was that p53-regulated DNA repair genes were activated by MSA. MSA treatment resulted in increased expression of p48XPE and Gadd45a proteins, component genes of the DNA repair branch of the p53 pathway (Figure 3). MSA activated DNA repair genes in the concentration range of 0.5-1.0 μM, while our earlier data showed DNA repair activation by SeMet in the concentration range of 10-20 μM. One possibility is that a small fraction of the input SeMet is metabolized to a lower-molecular-weight form that is the mediator of activity, and that MSA represents a more active form of selenium (14).

Of course, p53 modulation by selenium will have implications not only for DNA repair, but probably additional p53-regulated cellular functions. One important p53-mediated activity is apoptosis. The experiments reported herein utilized short-term (5-16 h) exposure to selenium, that is, only the immediate response of p53 to selenium was determined irrespective of the downstream cellular effects. At least in the short-term experiments shown herein, there was no evidence of cell death after selenium treatment as determined by trypan blue staining.

As a control for the current study, we used NaSel, which although widely used as a selenium source can also cause DNA strand-breaks and is therefore genotoxic as well as cytotoxic. NaSel enhanced p53 transactivation (Figure 1) as expected because p53 responds to genotoxic stress but also was observed with non-genotoxic selenium forms SeMet and MSA (25). NaSel caused phosphorylation of serines 20, 37, and 46, in contrast to the other selenium forms, although it too affected the reduction of cysteines 275/277 sulfhydryls (Figure 2). Phosphorylation of specific serine residues appears to be a distinctive feature of genotoxic selenium in the form of NaSel (Figure 2). We did not observe any effect on DNA repair by NaSel (0.5-1 μM; results not shown). Because of the short-term experimental design, apoptosis or other cell death were not observed (cell viability >95% by trypan blue staining).

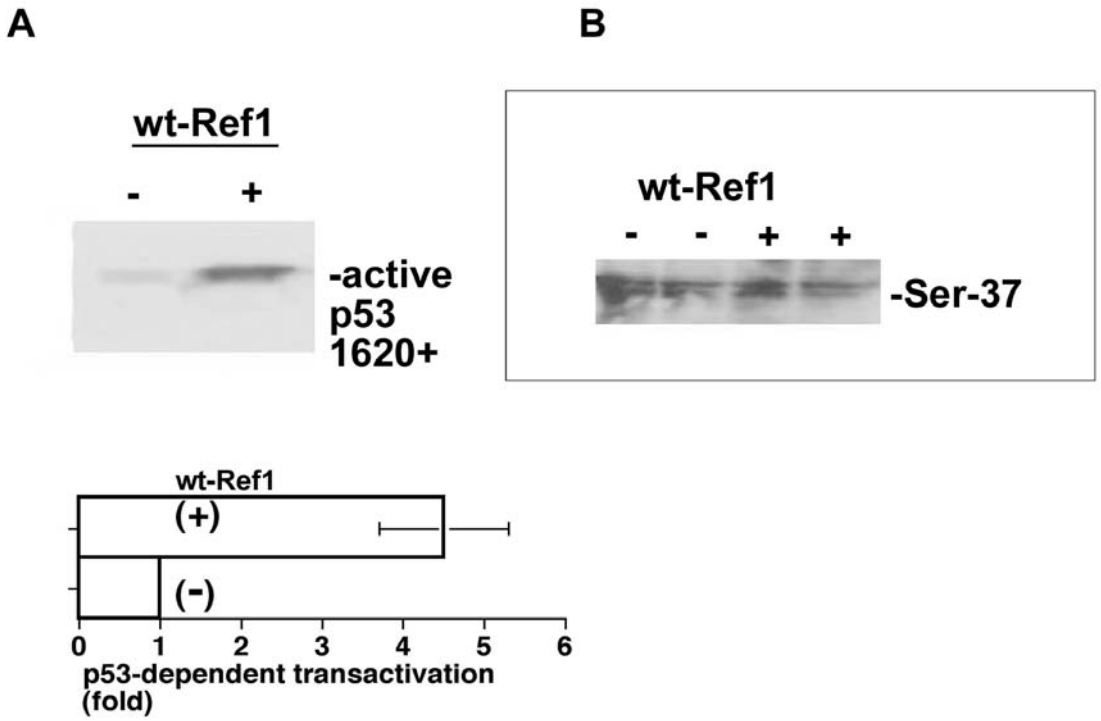

Figure 4. *Co-transfection of Ref1 and p53 recapitulates p53 activation similar to selenium. A) Cells were co-transfected with wild-type p53 and wild-type Ref1 vectors. Controls received the empty vector in place of Ref1. Active p53 protein was determined by Ab1620 (9). A separate series of experiments included the p53-dependent reporter gene pG13CAT in the transfections. Assays were conducted 48 h after transfection. Ref1 co-transfection increased levels of active p53 protein (upper panel) and increased p53-dependent trans-activation (lower panel). The level of p53-dependent transactivation in the presence of the Ref1 C65A mutant was below detection. B) Lack of effect on p53 phosphorylation in p53/Ref1 co-transfection experiments. Serine-37 is shown. Other serines and threonines likewise were not affected (not shown). Because Ref1 partially, but not completely, recapitulates selenium signaling to p53, we conclude that additional mediators are involved in selenium regulation of p53, such as cellular kinases that may be activated by individual selenium compounds.*

Role for Ref1 in selenium signaling to p53. The p53 response to SeMet was dependent on the redox factor Ref1, because blocking Ref1 by dominant-negative mutants or siRNA inhibitors blocked the p53 response (9). The current study using MSA adds to our previous finding in that MSA was found to induce Ref1 (APE/Ref1; Figure 3). These data are consistent with the reduction of cysteines 275/277 known to be mediated by Ref1 ((22); Figure 2). Co-transfection of Ref1 with p53 promoted p53 activation without a requirement for selenium supplementation (Figure 4). Ref1 drove p53 into an active conformation (expressing the Ab1620 epitope) and enhanced transactivation of a p53-dependent reporter gene (Figure 4). We conclude that besides SeMet, other selenium chemical forms also activate Ref1 and consequently p53. Ref1 is known to promote reduction of p53 cysteines 275/277 (25). Our finding of p53 phosphorylation in response to selenium stimulation (Figure 2) illuminates key differences between different selenium compounds in relation to p53 activation. One possibility is that co-transfection of p53 with Ref1 would recapitulate the phosphorylation patterns, for example if the redox control of cysteine residues and resultant conformation change affected accessibility of cellular kinases. Co-transfection of Ref1 together with p53 did not affect phosphorylation (Figure 4B) so it is likely that the selenium response involves activation of distinct cellular kinases that modulate p53 in concert with the Ref1-mediated modulation of p53 cysteine residues. It is noteworthy that the p53 cysteines regulated by Ref1 reside in the DNA-binding domain of p53 and are critical to sequence-specific DNA binding. Thus cysteines 275/277 are important but not sole mediators of selenium signaling to p53. Reduction of p53 cysteine sulfhydryl groups and p53 phosphorylation appear to be independent events.

Discussion

Implications of p53 as a selenium target. Our data shows that serum selenium concentration is a determinant of the setpoint for p53-mediated cellular functions (26). At physiological selenium concentrations, p53 controls basal expression of DNA repair genes XPC, p48XPE and Gadd45a (27). That p53 controls baseline expression of a subset of its downstream effector genes has been shown in several studies (20, 26, 28,

29). Because the bulk of nucleotide excision DNA repair (NER) occurs in the first few hours after DNA damage *e.g.* UV-irradiation, maintenance of DNA repair protein expression is critical to cell viability. Cells lacking p53, XPC, p48XPE, or Gadd45a genes lack an initial response to UV-damage compared to wild-type cells (17, 27, 30) and they exhibit a slow DNA repair phenotype. The NER defect in cells lacking any one of these genes is relatively modest (20-50% of wild-type). *Xeroderma pigmentosum* patients carrying XPC or XPE gene defects are cancer-prone despite the seemingly small magnitude of their defects (17, 27, 30, 31). Mice lacking Gadd45a genes are cancer-prone and exhibit an NER defect (16, 17). Cancers associated with modest NER defects are not limited to those with a UV-related etiology, *i.e.* skin cancers, because mice lacking XPC genes developed liver and lung cancers (32) and mice lacking Gadd45a genes developed mammary tumors (16).

The human population exhibits a range of DNA repair capacities that vary 2 to 3-fold even within the "normal range" (33). Those at the low end of the range are similar to XPC and XPE patients and are cancer prone (18). Persons at the upper end of the spectrum are more resistant to cancer development (33). The capacity of serum selenium concentrations to enhance p53 and its DNA repair subpathway is predicted to decrease the frequency of carcinogensis by decreasing the frequency of secondary mutations. Indeed, Gadd45a knockout mice showed increased mutagenesis of a germline reporter gene (16). It is surprising that DNA repair has not been more studied in the context of cancer prevention given these facts (33). In particular, selenium has been reported to inhibit field cancerization (34) a mechanism that seems hard to reconcile with heretofore envisioned mechanisms of cancer prevention *i.e.* elimination of irreparably-damaged or premalignant cells by apoptosis. Rather, field cancerization would seemingly require a molecular basis across an entire population of cells compared to simple elimination of occasional mutant cells. Of course, these viewpoints are not mutually exclusive. One mechanism by which field cancerization and prevention thereof may occur is by DNA repair. Inborn defects in DNA repair exemplified by *xeroderma pigmentosum* subgroups are cancer-prone providing evidence that DNA repair is central to cancer prevention, not only in UV-irradiated skin but involving internal tissues as well (32).

On the other hand, few would deny the importance of p53 as a determinant of additional cellular responses important in carcinogenesis prevention. One important response is apoptosis providing for elimination of cancerous or pre-cancerous cells. Indeed, apoptosis may be a recourse in irreparably damaged cells. Therefore, p53 is a key mediator of cellular life and death decisions of critical relevance to carcinogenesis. Our measurements of DNA repair are relatively short-term endpoints (3-48 h), while cell survival is more complex and reflects the balance between cell death and cell division over a longer period (2-12 days). The ability to eliminate irreparably damaged cells over the long term is presumably as important as the ability to repair DNA damage in the short term, as DNA repair pathways are not perfect. Indeed, error prone DNA polymerases may enhance cell survival at the cost of accruement of mutations (35). Endogenous DNA damage causes 10,000 lesions per day so one can argue that endogenous DNA damage is not trivial (36).

The data suggested that selenium may exert biologically important endpoints associated with p53. While SeMet modulated p53 and DNA repair in the 10-20 μM range, SeMet did not evoke demonstrable apoptosis or cell cycle arrest below 45 μM concentration, well above the physiological serum concentration of 1-5 μM (37). One reason why higher concentrations of SeMet may be required may be due to a rate-limiting bioconversion step, *i.e.* SeMet may need to be converted to a more active metabolite. For this reason we used MSA representing a low molecular weight metabolite of SeMet and other organoselenium forms including seleno-methylselenocysteine (14). Accordingly, MSA enhanced p53-dependent transactivation at lower concentrations than did SeMet (Figure 1). MSA led to elevated expression of DNA repair proteins of the p53 pathway, Gadd45a and p48XPE. A DNA repair response was observed similar to that reported for SeMet ((9), Figure 3). Other laboratories showed that apoptosis was induced by MSA in the concentration range of 2-5 μM, although longer times after selenium treatment were required to observe cell death (13, 14). Hence, physiological concentrations of MSA (1-5 μM) resulted in DNA repair and/or apoptosis even though apoptosis is a later event. Unlike SeMet which showed a wide separation of DNA repair *versus* apoptosis (10-20 μM *versus* >45 μM), MSA appears to evoke both responses in the 1-5 μM range. Of course cell type differences and factors *e.g.* levels of endogenous or exogenous DNA damage (36) also affect p53-mediated life and death responses. It is likely that p53 is an important mediator of selenium response, irrespective of cellular endpoints assayed. Our studies show that serum selenium concentration is a determinant of the setpoint for p53 activity, in turn affecting the critical balance between cell survival and cell death.

Implication of Ref1 as a co-activator of p53 in response to selenium. Our finding that MSA induces APE/Ref1 protein (Ref1; Figure 3) was not surprising in that Ref1 is known to interact with p53 and to promote reduction of p53 cysteine residue 275/277 sulfhydryl groups, which we observed for all three selenium compounds (Figure 2). The current data suggested that Ref1 and p53 activation may be endpoints common to diverse selenium compounds. In addition to being a positive regulator and cofactor for p53 *via* the Ref1 domain, the bifunctional protein APE/Ref1 can also participate in DNA repair independent of p53 (38). The apurinic endonuclease (APE) domain of APE/Ref1 is required for base excision DNA

repair (BER) the pathway for repair of 8-oxoguanine and other base damage. Interestingly, components of the BER pathway are also p53-regulated (39). There is, however, no evidence that selenium affects BER either through APE/Ref1 or p53, although this warrants further investigation.

APE/Ref1 is known to be induced by transcriptional and non-transcriptional mechanisms by DNA damage (38), however this is the first demonstration of its induction by non-genotoxic selenium in the form of MSA ((14); and Figure 3). Probably Ref1 protein like p53 is stabilized by reduction of key cysteine sulfhydryl groups (23). A role for thioredoxin reductase (TR) was suggested because 1) the redox function of Ref1 is thioredoxin-dependent (40); 2) a seleno-enzyme, TR activity was elevated 2 to 10-fold by SeMet treatment (41, 42); and 3) manipulation of TR levels affects p53 (43). The evidence for a role of Ref1 is 1) all three selenium compounds promoted a redox response of p53 cysteines 275/277 known to be mediated by Ref1 (Figure 2A); 2) a redox-dead mutant of Ref1 mutated at cysteine 65 blocked p53 response to selenium ((9) and Figure 3B); and 3) Ref1 when co-transfected with p53 recapitulated some of the selenium effect in the absence of selenium (Figure 4). Given the differences that we observed in the details of p53 activation by the three compounds (Figure 2), it is conceivable that different chemical forms of selenium may differ in their requirements for TR and/or Ref1.

For comparison to MSA we included NaSel, a genotoxic selenium compound. Although NaSel induced p53 as expected because it causes DNA damage, NaSel did not affect DNA repair of a UV-damaged reporter gene nor did it enhance cell survival (results not shown). NaSel was the only selenium compound tested that promoted serine phosphorylation or serines 20, 37 and 46 (Figure 2), which are associated with the activation of p53 for apoptosis. We did not observe apoptosis because of the short-term endpoints (5-16 h) after selenium treatment in our assays. MSA evidently activated p53 without phosphorylation of serine residues (Figure 2), but the role of p53 phosphothreonines is relatively unknown. One possibility is that phosphorylation of p53 serines *versus* threonines is a feature that discriminates between genotoxic signals to p53 (NaSel or DNA damage) and non-genotoxic signals to p53 (MSA or SeMet).

Conclusion. Our studies are novel in contrasting the actions of different selenium chemical forms on a single molecular determinant, p53. The use of different selenium forms may be applicable to future clinical trials in which selenomethionine is replaced with new-generation selenium compounds (3). Certain selenium forms may modify p53 for DNA repair while others may modify p53 for apoptosis (25). Our data also suggest that serum selenium concentrations may affect the setpoint for p53 activation and thereby contribute to DNA-damaging treatments including chemotherapy or radiation therapy.

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