

Blood Glutathione Peroxidase-1 mRNA Levels Can Be Used as Molecular Biomarkers to Determine Dietary Selenium Requirements in Rats

ROGER A. SUNDE,^{*,†,1} KEVIN M. THOMPSON,[†] JACQUELINE K. EVENSON,^{*,†}
AND BRITTA M. THOMPSON^{†,2}

**Department of Nutritional Sciences, University of Wisconsin, Madison, Wisconsin 53706; and [†]Department of Nutritional Sciences, University of Missouri, Columbia, Missouri 65211*

Transcript (mRNA) levels are increasingly being used in medicine as molecular biomarkers for disease and disease risk, including use of whole blood as a target tissue for analysis. Development of blood molecular biomarkers for nutritional status, too, has potential application that parallels opportunities in medicine, including providing solid data for individualized nutrition. We previously reported that blood glutathione peroxidase-1 (Gpx1) mRNA was expressed at levels comparable to major tissues in rats and humans. To determine the efficacy of using blood Gpx1 mRNA to assess selenium (Se) status and requirements, we fed graded levels of Se (0–0.3 µg Se/g as selenite) to weanling male rats. Se status was determined by liver Se concentration and selenoenzyme activity, and selenoprotein mRNA abundance in liver and blood was determined by ribonuclease protection analysis. Liver Se and plasma glutathione peroxidase-3 and liver Gpx1 activities indicated that minimal Se requirements were at 0.08 µg Se/g diet. When total RNA was isolated from whole blood, Gpx1 mRNA in Se-deficient rats decreased to 10% of levels in Se-adequate (0.2 µg Se/g diet) rats. With Se supplementation, blood Gpx1 mRNA levels increased sigmoidally to a plateau with a minimum Se requirement of 0.08 µg Se/g diet, whereas glutathione peroxidase-4 mRNA levels were unaffected. Similarly, Gpx1 mRNA in RNA

isolated from fractionated red blood cells decreased in Se-deficient rats to 23% of Se-adequate levels, with a minimum Se requirement of 0.09 µg Se/g diet. Additional studies showed that the preponderance of whole blood Gpx1 mRNA arises from erythroid cells, most likely reticulocytes and young erythrocytes. In summary, whole blood selenoprotein mRNA levels can be used as molecular biomarkers for assessing Se requirements, illustrating that whole blood has potential as a target tissue in development of molecular biomarkers for use in nutrition as well as in medicine. *Exp Biol Med* 234:1271–1279, 2009

Key words: erythrocytes; glutathione peroxidase-1; reticulocytes; ribonuclease protection analysis; transcripts; whole blood

Introduction

Measurement of mRNA transcript levels via high-throughput qRT-PCR and microarray technologies along with sophisticated bioinformatics is providing molecular biomarkers that are increasingly being developed and put into practice in medicine (1–5). These biomarkers perhaps offer more potential than biochemical biomarkers to identify presymptomatic phases of disease, predict patient outcome, identify clinically relevant patient subgroups, and increase understanding of disease mechanisms (1). Difficulty in obtaining target tissue samples, however, limits use of molecular biomarkers in both diagnosis and clinical research (5). Thus, genomics and transcriptomics investigators have begun to use peripheral blood as a “surrogate” tissue for biopsy and analysis; a recent review cites more than 40 human disease conditions that have been studied using gene-expression profiling on human peripheral blood cells (5). Development of molecular biomarkers for nutritional status based on peripheral blood, too, has potential application that parallels the opportunities in medicine, including providing solid data for individualized nutrition.

Our hypothesis is that assessment of selenium (Se)

This research was supported in part by USDA #95–37200-1799, by the University of Missouri Agricultural Experiment Station and the Food for the 21st Century Program, and by the University of Wisconsin Agricultural Experiment Station grant WIS04909.

¹ To whom correspondence should be addressed at Department of Nutritional Sciences, University of Wisconsin, 1415 Linden Drive, Madison, WI 53706. E-mail: sunde@nutrisci.wisc.edu

² Current address: Department Pediatrics, Baylor College of Medicine, Houston, TX 77030.

Received June 8, 2009.
Accepted July 29, 2009.

DOI: 10.3181/0906-RM-182
1535-3702/09/23411-1271\$15.00
Copyright © 2009 by the Society for Experimental Biology and Medicine

status provides an excellent model for development of less invasive molecular biomarkers for assessment of nutrient status. Glutathione peroxidase-1 (Gpx1) activity in rat liver drops dramatically in Se deficiency (6) and increases sigmoidally to a plateau with graded levels of dietary Se supplementation, thus providing a biochemical marker that was used to establish a dietary requirement of 0.1 $\mu\text{g Se/g}$ diet in the rapidly growing rat (7, 8). Discovery of additional selenoproteins, such as glutathione peroxidase-4 (Gpx4), selenoprotein P (Sepp1), deiodinase, thioredoxin reductase-1, selenoprotein W, and glutathione peroxidase-3 (Gpx3), provided further biochemical biomarkers of Se status that also indicate that minimum dietary Se requirements are $\leq 0.1 \mu\text{g Se/g}$ diet (9–14). Gpx1 mRNA levels in rat liver also drop dramatically in Se deficiency to 10–20% of Se-adequate levels (15), increase sigmoidally with increasing dietary Se, and reach well-defined plateaus and thus can serve as molecular biology-based biomarkers for Se status and for determination of Se requirements (9, 12, 16–20). Thus, we surveyed blood as a less invasive alternative tissue and found that Gpx1 mRNA and several other selenoprotein mRNAs were expressed at levels comparable to major tissues in rats (21) and humans (22); blood Gpx1 mRNA levels fall in Se deficiency similar to that in liver, at least in rats (21), suggesting that blood Gpx1 mRNA could also be used to assess Se status.

To determine the efficacy of using blood Gpx1 mRNA to assess Se status and requirements, we fed graded levels of dietary Se to young, rapidly growing rats and characterized the Se response using traditional biomarkers for Se status and using molecular biomarkers in liver, whole blood, and blood fractions. In addition, we also examined various fractions of blood to better understand the origin of Gpx1 mRNA in blood.

Materials and Methods

Reagents. Molecular biology reagents were purchased from Promega (Madison, WI), Invitrogen (Carlsbad, CA), or Sigma (St. Louis, MO). All other chemicals were of molecular biology or reagent grade.

Animals and Diets. *Experiment 1.* Male weanling rats ($n = 32$; 21 d old) weighing 55–72 g were obtained from Holtzman (Madison, WI) and housed individually in hanging wire mesh cages, following the care and treatment protocol approved by the Institutional Animal Care and Use Committee at the University of Missouri. Rats had free access to food and deionized water. The basal Se-deficient torula yeast-based diet (23) contained 0.008 $\mu\text{g Se/g}$ diet as determined by neutron activation analysis (24) and was supplemented with 100 mg/kg of all-rac- α -tocopherol acetate to ensure prevention of liver necrosis and supplemented with 4 g/kg D,L-methionine (U.S. Biochemical, Cleveland, OH) to ensure adequate growth, as described previously (16, 18). The rats were allocated randomly to treatment groups and supplemented with graded levels of Se

(0, 0.02, 0.05, 0.075, 0.1, 0.15, 0.2, or 0.3 $\mu\text{g Se/g}$) as Na_2SeO_3 for 28 d (4 rats/treatment). Body weight was measured biweekly, and rats were killed on d 28.

Experiment 2. Male weanling rats ($n = 6$; 21 d old) were obtained from Holtzman and treated as in Experiment 1. Rats were fed the basal Se-deficient diet supplemented with 0 or 0.2 $\mu\text{g Se/g}$ diet as Na_2SeO_3 for 28 d (3 rats/treatment).

Experiment 3. Male weanling rats ($n = 6$; 21 d old) were obtained from Holtzman and treated as in Experiment 1. Rats were fed the basal Se-deficient diet supplemented with 0.2 $\mu\text{g Se/g}$ diet as Na_2SeO_3 . On d 25, one group ($n = 3$) was anesthetized with ether, bled (1.5% of body weight) by cardiac puncture with a heparinized syringe, and allowed to recover; a sham group ($n = 3$) was also anesthetized and subjected to cardiac puncture, but no blood was drawn. Both groups were killed on d 28.

Tissue Analysis. On d 28, rats were anesthetized with ether and blood was collected in an EDTA-coated syringe. Aliquots of whole blood were quickly mixed with TRI Reagent BD (Molecular Research Center, Cincinnati, OH; typically 2 ml whole blood/7.5 ml TRI Reagent BD) and frozen at -80°C for RNA isolation. As described previously (7–9, 12, 17–20), whole blood and livers were collected and processed to determine tissue selenoenzyme activity and liver Se concentrations. Neutron activation analysis was used to determine liver and diet Se concentrations (24).

Blood Cell Fractionation and Counting. *Histopaque density gradients.* In Experiment 1, 5 ml of freshly drawn blood was layered over 5 ml of Histopaque-1083 (Sigma) and spun ($1,000 \times g$, 15 min) following the manufacturer's protocol. Three blood fractions were separated: red blood cells (RBCs), leukocytes, and plasma. RBCs and leukocytes were washed once in saline phosphate buffer. Guanidinium isothiocyanate was immediately added to the leukocyte and RBC fractions and frozen at -80°C for RNA isolation.

Discontinuous Percoll Gradient. In Experiment 2, blood cells were separated by discontinuous density gradient fractionation using Percoll gradients (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol and as described previously (25, 26). The gradient was formed by mixing an iso-osmotic stock of Percoll (9 volumes Percoll: 1 volume 1.5 M NaCl, pH 7.4) with 0.15 M NaCl (pH 7.4). This stock was used to make five density layers, 2 ml/layer, of densities 1.069, 1.085, 1.093, 1.099, and 1.105 g/ml. Density gradient fractionation was done in duplicate, one for RNA isolation and the other for characterization of cells within each fraction. Two milliliters of freshly drawn blood was first centrifuged ($1,400 \times g$, 15 min), and plasma was removed and replaced with an equivalent volume of saline (pH 7.4). The reconstituted blood (2 ml) was then carefully layered on the discontinuous gradient and centrifuged ($900 \times g$, 20 min, 21°C). After centrifugation, the five fractions were collected separately

and the cells washed once. One set of fractions was added to TRI Reagent BD (1 ml/3.25 ml TRI Reagent BD), mixed, and immediately frozen at -80°C , while differential cell counts were determined on the other set.

To facilitate quantitative isolation of RNA from these fractions, each fraction was mixed with 1.6 ml carrier whole blood that was freshly isolated from 6 second-generation Se-deficient male rats that had been fed a Se-deficient diet for 5–9 months (27). RNA was isolated as described below.

Cell Counting. Differential cell counts were determined by depositing a small aliquot of cells on a glass slide in a monolayer using a Cytospin-3 centrifuge (5 min, $250 \times g$; Shandon, Pittsburgh, PA). The cells were stained using a Wescor 7100 slide stainer (Wescor, Logan, UT) and evaluated under a light microscope. Reticulocyte cells were stained with new methylene blue (Sigma) according to the manufacturer's directions. Reticulocytes were defined as red blood cells containing two or more blue-stained granules (28). Nucleated cells from the whole blood and the leukocyte fraction were enumerated using a Coulter Counter (Model ZBI, Coulter Electronics, Hialeah, FL). In Experiment 3, cell counting was conducted by the MU Research Animal Diagnostic and Investigative Laboratory.

RNA Isolation. Liver and Histopaque Fraction RNA. RNA was isolated from 75 mg of liver or the Histopaque fractions as described by Chomczynski and Sacchi (29). The final RNA pellets were suspended in DEPC-treated water.

Blood RNA. RNA in whole blood and fractionated blood cells was isolated using TRI Reagent BD (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. The final RNA pellets were suspended in DEPC-treated water. RNA was quantitated spectrophotometrically by A_{260} ($\epsilon = 25 \text{ ml} \times \text{mg}^{-1} \times \text{cm}^{-1}$), and protein contamination was assessed by A_{260}/A_{280} . Ratios for liver and blood ranged from 1.80 to 2.07.

Ribonuclease Protection Analysis (RPA). RPA was conducted as previously described (18, 30). For each RNA sample, the Gpx1, Gpx4, and Sepp1 mRNA signals were normalized to the Gapdh mRNA signal.

Statistical Analysis. Data are presented as mean $\pm$ SEM; for Experiment 1, $n = 4/\text{treatment}$; for Experiments 2 and 3, $n = 3/\text{treatment}$. All data with three or more treatments were analyzed by one-way analysis of variance (ANOVA), and differences between means were assessed by Duncan's multiple range analysis ($P < 0.05$), with Kramer's modification for unequal class sizes (31). When variance equality was significant, as tested by Bartlett's test ($\alpha = 0.05$), significant differences between means were assessed instead by Scheffé's F-test. For Experiments 2 and 3, the unpaired student's t-test was used to compare two treatments. For all tests, $P < 0.05$ was considered significant. The plateau breakpoint for each Se response curve, defined as the intersection of the line tangent to the point of steepest slope and the plateau, was calculated as described previously (16–18) using sigmoidal or hyperbolic

regression analysis (Sigma Plot, Jandel Scientific) to estimate the minimum dietary Se necessary to obtain plateau responses.

Results

Experiment 1. Initial rat weights averaged 61.4 ± 0.7 g. There was no significant effect of dietary Se level on growth at any time during the study, with an average increase of 7.45 g/d, and final body weights averaged 270 ± 4 g (data not shown). In addition, there were no significant differences in final liver wet weights or liver protein concentrations, and no gross signs of liver necrosis were detected (data not shown). Thus, in these studies, as in previous studies (9, 12, 17), neither Se deficiency nor Se supplementation affected growth.

Traditional Biomarkers of Selenium Status. Liver Se concentrations in rats fed the basal diet were 6% of levels in Se-adequate ($0.2 \mu\text{g Se/g diet}$) rats, showing that these rats were Se deficient (Suppl. Fig. S1A). Se supplementation resulted in a sigmoidal response in liver Se concentration, with a plateau breakpoint at $0.08 \mu\text{g Se/g diet}$ (Table 1). In Se-deficient rats, plasma Gpx3, RBC Gpx1, liver Gpx1, and liver Gpx4 activities decreased to 6%, 1%, 24%, and 28%, respectively, of levels in Se-adequate rats (Suppl. Fig. S1B–E). The corresponding plateau breakpoints of the Se response curves (Table 1) were virtually the same as in previous studies as well, showing that the animals in this study were similar to previous studies.

Liver Selenoprotein mRNA Abundance. As in previous studies, RPA readily detected liver Gpx1, Gpx4, and Sepp1 mRNA (Fig. 1), and, as in previous studies, only Gpx1 mRNA was regulated by dietary Se, decreasing to 19% of levels in rats fed $0.2 \mu\text{g Se/g diet}$ (Suppl. Fig. S2A). In contrast, mRNAs for liver Gpx4 ($P = 0.48$) and Sepp1 ($P = 0.10$) (Suppl. Fig. S2B, C) were not regulated by dietary Se status. Gapdh mRNA levels were also not regulated by dietary Se status ($P = 0.42$, data not shown). Corresponding plateau breakpoints of Se response curves for the liver mRNAs are given in Table 1.

Whole Blood Selenoprotein mRNA Abundance. When RPA was used to quantitate Gpx1, Gpx4, and Gapdh mRNA in whole blood, we observed a similar pattern of Se regulation, with only Gpx1 mRNA decreased by Se deficiency (Fig. 1). RPA analysis and counting showed that whole blood Gpx1 mRNA levels in Se-deficient rats were 10% of the levels in rats fed $0.2 \mu\text{g Se/g diet}$ (Fig. 2A). With Se supplementation, whole blood Gpx1 mRNA levels increased with a plateau breakpoint at $0.08 \mu\text{g Se/g diet}$. In rats fed $0.3 \mu\text{g Se/g diet}$, whole blood Gpx1 mRNA levels were 32% higher but not significantly different from levels in rats fed $0.2 \mu\text{g Se/g diet}$. Gpx4 mRNA levels were not regulated by dietary Se (Suppl. Fig. S3A) ($P = 0.98$), and Sepp1 mRNA in total RNA from whole blood was not detected with the probes used in these studies.

RBC and Leukocyte Selenoprotein RNA. To begin

Table 1. Se Requirement Hierarchy in Growing Rats^a

Biomarker	Minimum requirement ^b (μg Se/g)	Extent of regulation ^c
Conventional biomarkers		
Growth	<0.008	Low ($P = 0.06$)
Liver Gpx4 activity	0.05	High ($P < 0.02$)
Plasma Gpx3 activity	0.08	Very high ($P < 0.0001$)
Liver Se concentration	0.08	Very high ($P < 0.0001$)
Liver Gpx1 activity	0.08	Very high ($P < 0.0001$)
RBC Gpx1 activity	0.11	High ($P < 0.0001$)
Molecular biomarkers		
Liver Gpx4 mRNA	<0.008	Low ($P = 0.48$)
Liver Sepp1 mRNA	<0.008	Low ($P = 0.10$)
WB Gpx4 mRNA	<0.008	Low ($P = 0.98$)
RBC Gpx4 mRNA	<0.008	Low ($P = 0.86$)
Liver Gpx1 mRNA	0.06	High ($P < 0.0001$)
WB Gpx1 mRNA	0.08	Very high ($P < 0.0002$)
RBC Gpx1 mRNA	0.09	High ($P < 0.002$)
Leuk Gpx1 mRNA	0.10	High ($P < 0.0001$)

^a Se, selenium; Gpx4, glutathione peroxidase-4; Gpx3, glutathione peroxidase-3; Gpx1, glutathione peroxidase-1; RBC, red blood cell; WB, whole blood; Leuk, leukocyte.

^b Minimum dietary Se requirement for the growing rat as determined for each indicated conventional biomarker in liver, plasma, and RBCs or each molecular biomarker in total RNA isolated from liver, WB, RBC fraction, or Leuk fraction. Requirements are the minimum dietary selenium necessary for the indicated parameter to reach plateau levels when Se-adequate weanling rats are fed these diets from weaning, as determined by breakpoint analysis as described in the text.

^c Susceptibility to Se regulation of the indicated biomarker in Se-deficient rat tissue in Se-deficient versus Se-adequate (0.20 μg Se/g diet) rats: very high = <10.9% of Se-adequate; high = 11–40.9% of Se-adequate; moderate = 41–70% of Se-adequate; low = >70% of Se-adequate. Significance (P value) of regulation indicated in parentheses.

to understand the origin of whole blood mRNA, blood was separated into RBCs, leukocytes, and plasma by density gradient centrifugation. Differential cell counting revealed that, on a relative basis, the resulting RBC fraction contained 95% mature erythrocytes with negligible leuko-

cyte cell contamination (less than 0.03% of the total cells). The resulting leukocyte fraction contained 65% lymphocytes + monocytes cells with 35% erythroid cells (data not shown). Reticulocytes were not detected in the stained in leukocyte fraction, most likely due to limited cells or

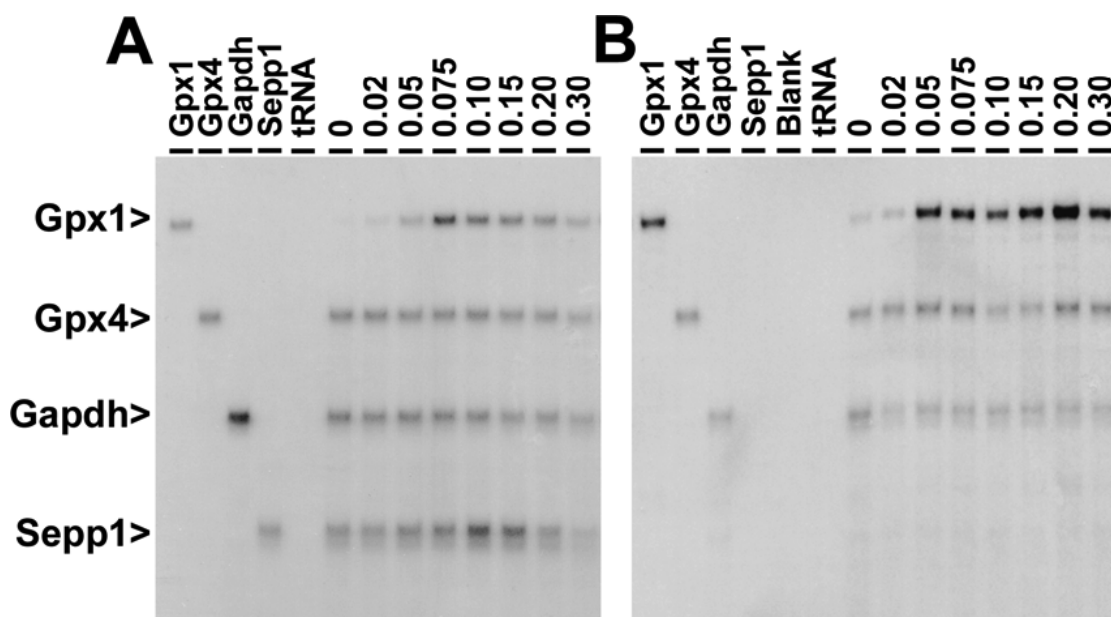


Figure 1. Ribonuclease protection analysis (RPA) of selenoprotein mRNA. (A) Liver. (B) Blood. Total RNA (10 μg), isolated from rats supplemented with the indicated levels of Se for 28 d, was analyzed for Gpx1, Gpx4, Gapdh, and Sepp1. A Se-adequate RNA sample was hybridized individually with 2× concentration of a single probe (lanes 1–4). Yeast tRNA (lanes A5 and B6) was analyzed with the probe mixture as a negative control. The figure is representative of four autoradiographs.

residual contamination. Coulter counting revealed that the leukocyte fraction contained 5.7×10^6 nucleated cells. The plasma fraction contained insufficient cells, confirming that mRNA in whole blood was not arising from plasma. When total RNA was isolated from the density gradient fractions, the RBC and leukocyte fractions contained $76 \pm 4\%$ and $13 \pm 1\%$, respectively, of the total RNA isolated from whole blood RNA (data not shown), with an apparent loss of 11% most likely due to RNA degradation during the fractionation process.

The Gpx1 mRNA level in total RNA from the RBC fraction isolated from Se-deficient rats was 23% of levels in Se-adequate rats (Fig. 2B). Gpx1 mRNA increased sigmoidally with increasing dietary Se, with a plateau breakpoint at $0.09 \mu\text{g Se/g}$ diet and no increase in Gpx1 mRNA in rats fed $0.3 \mu\text{g Se/g}$ diet, compared with rats fed $0.1\text{--}0.2 \mu\text{g Se/g}$ diet. In contrast, there was no effect of dietary Se on Gpx4 mRNA in total RNA from the RBC fractions ($P = 0.86$, Suppl. Fig. S3B). Although the Gpx1 mRNA detected in total RNA from the leukocyte fraction was considerably less than that isolated from the RBC fraction, Se-deficient Gpx1 mRNA was 11% of Se-adequate levels with a plateau breakpoint at $0.10 \mu\text{g Se/g}$ diet (Fig. 2C); Gpx4 mRNA in the leukocyte fraction only decreased in Se deficiency to 60% of Se-adequate levels (data not shown).

Collectively, calculation of Gpx1 mRNA in Se-adequate rats ($0.1\text{--}0.3 \mu\text{g Se/g}$ diet) in the RBC and leukocyte fractions, taking into account total RNA recovery in individual samples, indicated that RBC Gpx1 mRNA accounted for 71% of whole blood Gpx1 mRNA (280 ± 23 versus 396 ± 37 dpm/ml blood), while the leukocyte fraction accounted for only 25% of whole blood Gpx1 mRNA. Because erythroid cells were also detected in the leukocyte fraction, these cells may have contributed to the Gpx1 mRNA detected in this fraction. RBC and leukocyte Gpx4 mRNA accounted for 113% and 20%, respectively, of whole blood Gpx4 mRNA.

Experiment 2. Traditional Biomarkers of Se Status. There was also no effect of dietary Se supplementation on growth, and the rats grew at an average of 7.5 g/d over the 28-d supplementation period (data not shown). Liver Gpx1, plasma Gpx3, RBC Gpx1, and liver Gpx4 activities in rats fed the Se-deficient diet were 1%, 5%, 30%, and 53%, respectively, of levels in Se-adequate rats (data not shown), showing that these rats were Se deficient.

Liver and Whole Blood Selenoprotein mRNA Abundance. Liver Gpx1 mRNA levels in Se-deficient rats were 20% of Se-adequate levels, but liver Gpx4 and Sepp1 mRNA levels, as in Experiment 1, were not decreased in Se deficiency (data not shown). Whole blood Gpx1 mRNA levels in Se-deficient rats were also 21% of Se-adequate levels, whereas whole blood Gpx4 mRNA levels were not significantly affected by Se status (Fig. 3).

Blood Fractionation. To better localize the cellular origin of Gpx1 mRNA detected in total RNA from whole

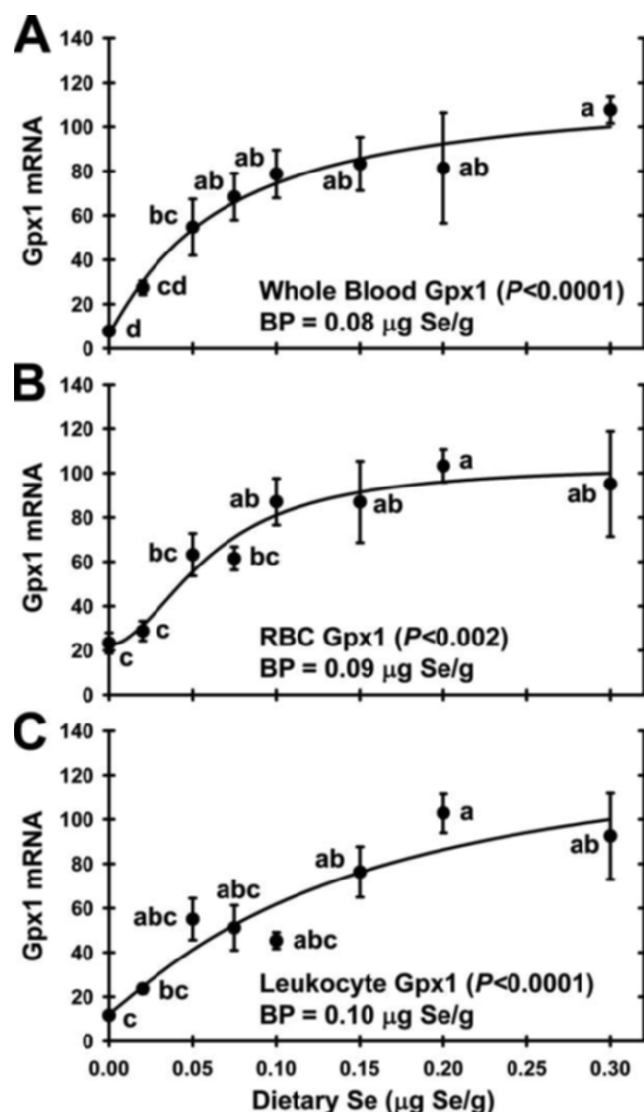


Figure 2. Effect of dietary Se on blood selenoprotein transcript abundance. (A) Whole blood Gpx1; (B) RBC Gpx1; (C) Leukocyte Gpx1. Values are mean $\pm$ SEM; $n = 4/\text{diet}$. The level of significance by ANOVA is indicated in each panel; values with a common letter are not significantly different ($P < 0.05$). The calculated plateau breakpoint (BP) for each Se response curve is also indicated.

blood, washed blood cells from 2 ml of blood from each Se-adequate rat were separately subjected to discontinuous Percoll-gradient fractionation, as described in the Materials and Methods section. One set of the resulting fractions was immediately mixed with TRI Reagent BD for later RNA isolation and analysis, and one set was stained and counted to determine distribution of blood cells in each fraction. As described in the Materials and Methods section, each fraction was combined with 1.6 ml of whole blood in TRI Reagent BD from second-generation Se-deficient rats to provide carrier RNA, and total RNA was isolated from each fraction. RNA recovery from the carrier 1.6 ml whole blood alone averaged $42.8 \pm 3.4 \mu\text{g}$, compared with an average of $48.4 \pm 2.7 \mu\text{g}$ for all of the Percoll fractions, and there was

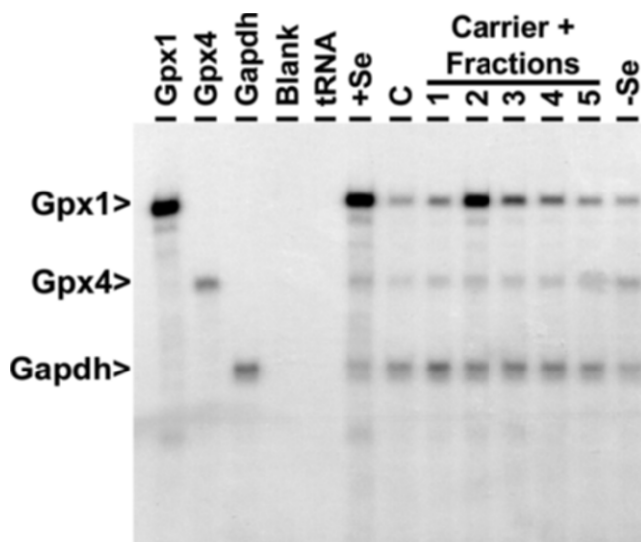


Figure 3. Ribonuclease protection analysis (RPA) of selenoprotein mRNA in fractionated blood. Total RNA was isolated from whole blood from Se-deficient (–Se), Se-adequate (+Se, 0.2 µg Se/g diet), or second-generation Se-deficient (C for carrier) rats or from Percoll-gradient fractions combined with carrier blood (fractions 1–5), as described in the text. Total RNA (10 µg) was analyzed for Gpx1, Gpx4, and Gapdh. A Se-adequate RNA sample was hybridized with control probes (lanes 1–5), as described in Figure 1. The figure is representative of three autoradiographs.

no significant difference in RNA yield of different fractions or due to Se status ($P = 0.94$, data not shown).

The resulting RNA samples (from fraction RNA plus carrier RNA) were subjected to RPA to quantitate selenoprotein mRNA levels (Fig. 3). Gpx1 mRNA levels in carrier whole blood alone were 19% of Se-adequate levels. When the level of carrier blood Gpx1 mRNA was subtracted from the Gpx1 mRNA detected in each fraction, it was clear the preponderance (82–85%) of the Gpx1 mRNA detected by RPA was located in fractions 2 and 3 (Fig. 4); in one rat, 44% and 40% of the Gpx1 mRNA was present in fraction 2 and 3, respectively, whereas 78% was in just fraction 2 for the second rat and 74% was just in fraction 3 for the third rat. Coulter counting found that fractions 1 and 2 contained similar amounts of nucleated cells, 3 and 3.6×10^6 cells/ml of blood, respectively, while fractions 3, 4, and 5 contained virtually no nucleated cells. Fraction 1 contained 2.7×10^6 lymphocytes, 3×10^5 monocytes, 6×10^3 polymorphonuclear cells, and 9.2×10^5 erythroid cells, and fraction 2 contained 3.3×10^6 lymphocytes, 2.5×10^5 monocytes, no polymorphonuclear cells, and 2×10^6 erythroid cells. Fraction 5 in this discontinuous gradient system contained the vast majority of mature erythrocytes (26). Thus, whereas fractions 1 and 2 contained similar numbers of leukocytes, there was negligible Gpx1 mRNA in fraction 1, indicating that the preponderance of the Gpx1 mRNA was not arising from leukocytes. Differential cell counting (Fig. 4) also showed that lymphocytes and monocytes declined from 61% and 7%, respectively, of the cells in fraction 1, to 2% and 0%,

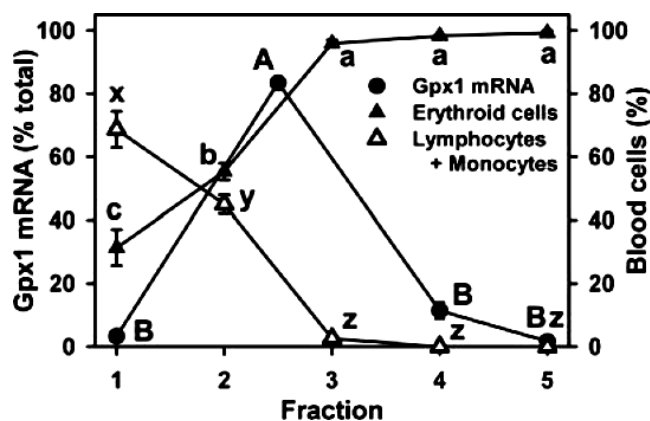


Figure 4. Effect of Percoll-gradient fractionation on Gpx1 mRNA abundance and on erythrocytes and leukocytes in the resulting fractions. Se-adequate rat blood was fractionated on Percoll gradients, and resulting fractions were analyzed, as described in the text. Shown is the percentage of total recovered Gpx1 mRNA detected by RPA in each fraction (left) and the percentage of erythroid cells and lymphocytes + monocytes in each fraction (right). Values are means $\pm$ SEM, $n = 3$; values with a common letter are not significantly different by ANOVA ($P < 0.05$). The Gpx1 mRNA value at fraction 2.5 is the sum of fractions 2 and 3, as described in the text.

respectively, of the cells in fraction 1, whereas erythroid cells increased from 31% in fraction 1 to >96% in fractions 3–5. In this experiment, new methylene blue staining was unable to distinguish reticulocytes from mature erythrocytes, perhaps due to Percoll interference.

Experiment 3. Traditional Biomarkers. Growth up to d 25 was similar to that in Experiments 1 and 2 (data not shown). Bleeding significantly decreased blood hemoglobin from 129 ± 3 to 119 ± 3 mg/ml, decreased hematocrit from 45 ± 1 to $41 \pm 1\%$, and increased reticulocyte count from 17 ± 5 to 42 ± 13 cells/1,000 cells but did not alter leukocyte counts 10.9 ± 1.3 versus 10.8 ± 1.6 thousand cells/ μ l, respectively. It is important that on d 28 neither plasma Gpx3 activity nor RBC Gpx1 activity was altered by bleeding (data not shown).

Molecular Biomarkers. Yield of total RNA isolated from whole blood, however, more than doubled after bleeding from 53 ± 3 µg/ml to 118 ± 9 µg/ml. RPA of total RNA isolated from whole blood revealed that the increase in whole blood reticulocytes and total RNA was accompanied by 288 and 217% increases in Gpx1 and Gpx4 mRNA, respectively (Fig. 5), whereas Gapdh mRNA decreased 27%.

Discussion

Assessing Se status and Se requirements in humans using molecular biomarkers has potential but obviously will need to use a less-invasive source of mRNA than liver. Thus, we surveyed blood in rats as a source of mRNA and found that several selenoprotein mRNAs were expressed in blood at levels comparable to levels in the major organs of the rat (21). We initiated the present studies to better characterize the effect of Se status on Gpx1 mRNA in rat

blood. We were able to reproducibly recover ~ 50 μg total RNA/ml of whole rat blood regardless of Se status. Whole blood and RBC Gpx1 mRNA levels decreased in Se-deficient rats to 10–20% of levels found in total RNA from Se-adequate rats and increased sigmoidally with increasing dietary Se to a plateau with plateau breakpoints at 0.08–0.09 μg Se/g diet. Similar to liver, Gpx4 mRNA was not decreased by Se deficiency in whole blood total RNA, and Gapdh mRNA was also similarly not decreased by Se deficiency. In these studies, we were not able to detect blood Sepp1 mRNA using RPA probes.

Fractionation of blood into RBC, leukocyte, and plasma fractions in Experiment 1 using Histopaque density gradients revealed that 71% of the Gpx1 mRNA found in whole blood could be recovered in the RBC fraction in spite of negligible leukocyte contamination, clearly indicating that the majority of detected Gpx1 mRNA arose from erythroid cells rather than leukocytes. In Experiment 2, fractionation of blood cells into five fractions using Percoll-gradient fractionation resulted in the preponderance of the Gpx1 mRNA in fractions rich in reticulocytes and young erythrocytes rather than leukocyte (fraction 1) or mature erythrocyte fractions (fraction 5). Lastly, in Experiment 3, prebleeding of rats 3 d before resulted in a 288% increase in Gpx1 mRNA along with a 240% increase in reticulocytes and a 440% increase in total RNA yield but without any increase in leukocytes. Gapdh mRNA, which is associated with the leukocyte fraction (21), actually decreased 27% in the bled rats. Thus, it is clear that the preponderance of Gpx1 mRNA found in total RNA isolated from whole blood in rats arises from erythroid cells and most likely reticulocytes and young erythrocytes. Young (lower density) erythrocytes are reported to have 9% more Gpx1 activity, and mature (higher density) erythrocytes are reported to have 14% less Gpx1 activity, compared with unfractionated erythrocytes in young rats (32), suggesting that both activity and mRNA are lost as erythrocytes mature.

These fractionation studies match well with a number of recent studies on mRNA in peripheral blood. Microarray and qRT-PCR studies on human as well as rodent mRNA in peripheral whole blood indicate that as much as 70% of total RNA isolated from whole blood is hemoglobin mRNA (33), demonstrating that erythroid cells are clearly the predominant source of RNA isolated from whole blood. Recent studies have found that total RNA from human RBCs resembles typical eukaryotic RNA, with a 5S–80S sedimentation distribution similar to sedimentation distributions in avian (nucleated) erythrocytes; this RNA contains the standard 18S- and 28S-rRNA bands indicative of protein synthesis (34). Furthermore, genes identified in total RNA included transcripts of genes encoding initiation, activation, and regulation of transcription and translation and included RNA-stabilizing factors and antiapoptotic proteins (34). Other studies using microarray analysis have shown that peripheral blood cells share more than 80% of the transcriptome with each of nine tissues studied (brain,

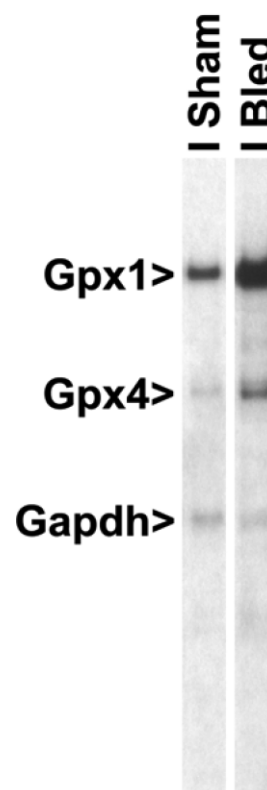


Figure 5. Ribonuclease protection analysis (RPA) of selenoprotein mRNA in whole blood from sham-bled and bled rats. Total RNA (10 μg) isolated from Se-adequate rats (0.2 μg Se/g diet). On d 25, one group ($n=3$) was bled (1.5% of body weight) by cardiac puncture and allowed to recover, and a sham control group ($n=3$) was subjected to cardiac puncture but no blood was drawn. Rats were killed on d 28, and blood total RNA was analyzed for Gpx1, Gpx4, and Gapdh. Shown lanes are from the same gel and are representative of three autoradiographs.

colon, heart, kidney, liver, lung, prostate, spleen, and stomach), and estimates are that the blood transcriptome contains 16,000–20,000 transcripts (35). Thus, it is likely that a number of transcripts in whole blood RNA have potential as molecular biomarkers for status of other nutrients.

The minimum requirement based on liver Gpx1 mRNA as a molecular biomarker in Experiment 1 is similar to minimum requirements determined in previous studies based on Gpx1 mRNA levels as determined by Northern blotting (9, 16, 17), by RPA (12, 18), and by qRT-PCR (20). At this point, it is too early to discern whether the minimum Se requirement based on whole blood or RBC Gpx1 mRNA (0.08–0.09 μg Se/g) is consistently and significantly higher than requirements based on liver Gpx1 mRNA, which in this study and in previous studies ranged between 0.05 and 0.07 μg Se/g diet (9, 16, 17, 20). We recently reported that the minimum dietary Se requirements based on mRNA levels of Selh and Sepw1 as well as Gpx1 in liver, kidney, and muscle, all ranged between 0.03 and 0.07 μg Se/g diet and were less than those based on biochemical biomarkers, such as Gpx1 and Gpx3 enzyme activity (20). In testis,

however, we found that Se requirements for Gpx1 activity were less than for Gpx1 mRNA (36), indicating that there may be distinct patterns of Se regulation due to targeted trafficking of Se or due to separate metabolic pools of Se. Stage of the life cycle can also affect Se requirements, because adult pregnant rats (18) and old female rats (37) have lower dietary Se requirements than young, rapidly growing rats (16). Whole blood RNA may represent an integration of Se status over some portion of erythropoiesis that could offer a special advantage to nutritionists, because it potentially would integrate nutrient status over a longer period of time than more conventional biomarkers, such as plasma Se.

In summary, the studies reported here show that blood Gpx1 mRNA levels can also be used to determine minimum dietary Se requirements in rats. Peripheral blood clearly has potential for development of molecular biomarkers in nutrition, as well as in medicine. At this point, it is not likely that selenoprotein-based molecular biomarkers will be useful in discriminating between Se status in populations like the United States versus populations with marginally lower Se intake like in Europe (22), but they may be useful for more severely Se-deficient populations (38). Future discovery microarray studies, however, have the potential to identify nonselenoprotein transcripts, perhaps related to Se metabolism, that might discriminate between the Se-marginal and Se-adequate populations or that might differentiate between individuals who will benefit versus will be adversely affected by Se supplementation (39). Such mRNAs, if identified, would illustrate the powerful contribution that whole blood molecular biomarkers could make to the study of nutrition.

1. Bell J. Predicting disease using genomics. *Nature* 429:453–456, 2004.
2. Winegarden N. Microarrays in cancer: moving from hype to clinical reality. *Lancet* 362:1428, 2003.
3. Gerszten RE, Wang TJ. The search for new cardiovascular biomarkers. *Nature* 451:949–952, 2008.
4. de Leon J. Pharmacogenomics: the promise of personalized medicine for CNS disorders. *Neuropsychopharmacology* 34:159–172, 2009.
5. Mohr S, Liew CC. The peripheral-blood transcriptome: new insights into disease and risk assessment. *Trends Mol Med* 13:422–432, 2007.
6. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. *Science (Washington, DC)* 179:588–590, 1973.
7. Hafeman DG, Sunde RA, Hoekstra WG. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *J Nutr* 104:580–587, 1974.
8. National Research Council. *Nutrient Requirements of Laboratory Animals* (3rd ed.). Washington, DC: National Academy Press, pp 7–37, 1978.
9. Lei XG, Evenson JK, Thompson KM, Sunde RA. Glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase are differentially regulated in rats by dietary selenium. *J Nutr* 125:1438–1446, 1995.
10. Yang JG, Hill KE, Burk RF. Dietary selenium intake controls rat plasma selenoprotein P concentration. *J Nutr* 119:1010–1012, 1989.
11. Bermano G, Nicol F, Dyer JA, Sunde RA, Beckett GJ, Arthur JR, Hesketh JE. Tissue-specific regulation of selenoenzyme gene expression during selenium deficiency in rats. *Biochem J* 311:425–430, 1995.
12. Hadley KB, Sunde RA. Selenium regulation of thioredoxin reductase activity and mRNA levels in rat liver. *J Nutr Biochem* 12:693–702, 2001.
13. Yeh JY, Vendeland SC, Gu Q, Butler JA, Ou BR, Whanger PD. Dietary selenium increases selenoprotein W levels in rat tissues. *J Nutr* 127:2165–2172, 1997.
14. Cohen HJ, Chovanec ME, Mistretta D, Baker SS. Selenium repletion and glutathione peroxidase: differential effects on plasma and red blood cell enzyme activity. *Am J Clin Nutr* 41:735–747, 1985.
15. Saedi MS, Smith CG, Frampton J, Chambers I, Harrison PR, Sunde RA. Effect of selenium status on mRNA levels for glutathione peroxidase in rat liver. *Biochem Biophys Res Commun* 153:855–861, 1988.
16. Weiss SL, Evenson JK, Thompson KM, Sunde RA. The selenium requirement for glutathione peroxidase mRNA level is half of the selenium requirement for glutathione peroxidase activity in female rats. *J Nutr* 126:2260–2267, 1996.
17. Weiss SL, Evenson JK, Thompson KM, Sunde RA. Dietary selenium regulation of glutathione peroxidase mRNA and other selenium-dependent parameters in male rats. *J Nutr Biochem* 8:85–91, 1997.
18. Sunde RA, Evenson JK, Thompson KM, Sachdev SW. Dietary selenium requirements based on glutathione peroxidase-1 activity and mRNA levels and other selenium parameters are not increased by pregnancy and lactation in rats. *J Nutr* 135:2144–2150, 2005.
19. Sunde RA, Raines AM, Barnes KM, Evenson JK. Selenium status highly-regulates selenoprotein mRNA levels for only a subset of the selenoproteins in the selenoproteome. *Biosci Rep* 29:329–338, 2009.
20. Barnes KM, Evenson JK, Raines AM, Sunde RA. Transcript analysis of the selenoproteome indicates that dietary selenium requirements in rats based on selenium-regulated selenoprotein mRNA levels are uniformly less than those based on glutathione peroxidase activity. *J Nutr* 139:199–206, 2009.
21. Evenson JK, Wheeler AD, Blake SM, Sunde RA. Selenoprotein mRNA is expressed in blood at levels comparable to major tissues in rats. *J Nutr* 134:2640–2645, 2004.
22. Sunde RA, Paterson E, Evenson JK, Barnes KM, Lovegrove JA, Gordon MH. Longitudinal selenium status in healthy British adults: assessment using biochemical and molecular biomarkers. *Br J Nutr* 99: S37–S47, 2008.
23. Knight SAB, Sunde RA. The effect of progressive selenium deficiency on anti-glutathione peroxidase antibody reactive protein in rat liver. *J Nutr* 117:732–738, 1987.
24. McKown DM, Morris JS. Rapid measurement of selenium in biological samples using instrumental neutron activation analysis. *J Radioanal Nucl Chem* 43:411–420, 1978.
25. Robertson A, Morrison JN, Wood AM, Bremner I. Effects of iron deficiency on metallothionein-I concentrations in blood and tissues of rats. *J Nutr* 119:439–445, 1989.
26. Lindena J, Sommerfeld U, Hopfel C, Wolkersdorfer R, Trautschold I. Enzyme activities in rabbit, guinea pig, rat and mouse blood cells after separation on a discontinuous percoll gradient. *Enzyme* 29:229–238, 1983.
27. Thompson KM, Haibach H, Sunde RA. Growth and plasma triiodothyronine concentrations are modified by selenium deficiency and repletion in second-generation selenium-deficient rats. *J Nutr* 125: 864–873, 1995.
28. Cavill I. The rejected reticulocyte. *Br J Haematol* 84:563–565, 1993.
29. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987.
30. Weiss Sachdev S, Sunde RA. Selenium regulation of transcript abundance and relative translational efficiency of glutathione peroxidase 1 and 4 in rat liver. *Biochem J* 357:851–858, 2001.

31. Steel RGD, Torrie JH. Principles and Procedures of Statistics. New York, NY: McGraw-Hill Book, 1960.
32. Murakami T, Takemori K, Shirasaka N, Yoshizumi H, Ito H. Cell age distribution of erythrocytes at the incidence of cerebral stroke in stroke-prone spontaneously hypertensive rats, and their glutathione peroxidase activity. *Biosci Biotechnol Biochem* 62:484–490, 1998.
33. Tian Z, Palmer N, Schmid P, Yao H, Galdzicki M, Berger B, Wu E, Kohane IS. A practical platform for blood biomarker study by using global gene expression profiling of peripheral whole blood. *PLoS ONE* 4:e5157, 2009.
34. Kabanova S, Kleinbongard P, Volkmer J, Andree B, Kelm M, Jax TW. Gene expression analysis of human red blood cells. *Int J Med Sci* 6: 156–159, 2009.
35. Liew CC, Ma J, Tang HC, Zheng R, Dempsey AA. The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. *J Lab Clin Med* 147:126–132, 2006.
36. Schriever SC, Barnes KM, Evenson JK, Raines AM, Sunde RA. Selenium requirements are higher for glutathione peroxidase-1 mRNA than Gpx1 activity in rat testis. *Exp Biol Med* 234:513–521, 2009.
37. Sunde RA, Thompson KM. Dietary selenium requirements based on tissue selenium concentration and glutathione peroxidase activities in old female rats. *J Trace Elem Med Biol* 23:132–137, 2009.
38. Xia Y, Hill KE, Byrne DW, Xu J, Burk RF. Effectiveness of selenium supplements in a low-selenium area of China. *Am J Clin Nutr* 81:829–834, 2005.
39. Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, Gaziano JM, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* 301:39–51, 2009.