Selenium Suppresses Leukemia through the Action of Endogenous Eicosanoids

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Abstract
Eradicating cancer stem-like cells (CSC) may be essential to fully eradicate cancer. Metabolic changes in CSC could hold a key to their targeting. Here, we report that the dietary micronutrient selenium can trigger apoptosis of CSC derived from chronic or acute myelogenous leukemias when administered at supraphysiologic but nontoxic doses. In leukemia CSC, selenium treatment activated ATM-p53-dependent apoptosis accompanied by increased intracellular levels of reactive oxygen species. Importantly, the same treatment did not trigger apoptosis in hematopoietic stem cells. Serial transplantation studies with BCR–ABL-expressing CSC revealed that the selenium status in mice was a key determinant of CSC survival. Selenium action relied upon the endogenous production of the cyclooxygenase-derived prostaglandins \( \Delta^{12} \)-PGJ2 and 15d-PGJ2. Accordingly, nonsteroidal anti-inflammatory drugs and NADPH oxidase inhibitors abrogated the ability of selenium to trigger apoptosis in leukemia CSC. Our results reveal how selenium-dependent modulation of arachidonic acid metabolism can be directed to trigger apoptosis of primary human and murine CSC in leukemia. Cancer Res; 74(14): 3890–901. ©2014 AACR.

Introduction
Leukemia is a hierarchical disease in which leukemia stem cells (LSC) occupy the apex and give rise to bulk leukemia cells that are responsible for the pathology of the disease. However, unlike LSCs, bulk leukemia cells are unable to initiate leukemia when transplanted into secondary recipients. Because of this property, LSCs represent a challenge to current therapy in that they can actively resist chemotherapy regimens and cause relapse of the disease (1, 2). In chronic myelogenous leukemia (CML), the BCR–ABL fusion protein generated from the 9;22 translocation is capable of generating LSCs when expressed in hematopoietic stem cells (HSC; ref. 3). Although current treatment using tyrosine kinase inhibitors (TKI) can block the production of bulk leukemia cells and lead to remission of CML, these agents do not affect LSCs (1). Therefore, new therapies are needed that target LSCs to prevent relapse following traditional TKI therapy.

Cyclopentenone prostaglandins (CyPG), \( \Delta^{12} \)-PGJ2 and 15d-PGJ2, are derived from the polyunsaturated fatty acid, arachidonic acid (ARA), via the sequential action of cyclooxygenases (COX) and PGD2 synthases (PGDS) followed by nonenzymatic conversion of PGD2 to \( \Delta^{12} \)-PGJ2 and 15d-PGJ2 (4, 5). We have recently demonstrated the ability of \( \Delta^{12} \)-PGJ2 and 15d-PGJ2 to selectively activate the p53-dependent pathway of apoptosis in LSCs, without affecting the (normal) HSCs in a murine model of CML and in an unrelated model of Friend virus–induced erythroleukemia (6). The importance of the COX pathway in leukemia has been highlighted in a few epidemiologic studies in which increasing incidences of leukemia were associated with nonsteroidal anti-inflammatory drugs (NSAID) consumption (7, 8), which led us to study strategies to increase endogenous CyPGs for treatment of leukemia. Previous studies have shown supplementation of mice or macrophages with selenium, a micronutrient that functions through insertion into redox-active selenoproteins, led to enhanced production of CyPGs as opposed to proinflammatory PGE2, TXA2, or PGF2\( \alpha \) through a process termed “eicosanoid class switching” (9, 10). Therefore, we hypothesized that supraphysiologic levels of selenium may play a key role in treating leukemia through the ability of endogenous CyPGs to target LSCs. In fact, low serum selenium levels have been noted in leukemic patients (11–16). On the other hand, use of selenium-cystine (as diselenodialanine) in leukemic patients decreased total leukocyte count, immature leukocytes in patients with CML and acute myelogenous leukemia (AML; ref. 17). Antileukemic effect of sodium selenite (Na2SeO3) was suggested to activate p53 to cause apoptosis (18–24). However, the mechanism by which selenium activates p53 is not well understood.

In addition to being the guardian of the genome, p53 also regulates glycolysis and aerobic respiration through oxidative
phosphorylation (OXPHOS) in cancer stem-like cells (CSC; ref. 25). This process is accompanied by an increase in the expression of *Tigar* (TPp53-inducible glycolysis and apoptosis regulator), Rn2b (Ribonucleotide-diphosphate reductase subunit RM2B), and Sco2 (synthesis of cytochrome oxidase-2) that induce cell-cycle arrest followed by reactive oxygen species (ROS)–dependent apoptosis of irreparable cells (26–28). However, p53 also upregulated an antioxidant response through a sestrin-dependent Nrf-2 activation pathway (29). Thus, by establishing complex regulatory networks involving temporally segregated responses, p53 can cooperate with redox changes to affect cellular metabolism and survival involving intricate control of intracellular ROS levels, resulting in diverse outcomes. In fact, the sensitivity of LSCs to agents that target p53 and apoptosis of LSCs. In addition, treatment of blood samples from human patients with AML and blast-crisis CML with lipid extracts (LE) from selenium-supplemented macrophages induced apoptosis in CD34+CD38−CD123+ LSCs, which was blocked by NSAIDs. The proapoptotic effects of selenium were, in part, related to exacerbated oxidative stress in LSCs that involved NADPH oxidases, particularly Nox1. In contrast, selenium treatment did not affect normal HSCs, establishing complex regulatory networks involving temporal-segregated responses, p53 can cooperate with redox changes (29). Thus, by establishing complex regulatory networks involving temporally segregated responses, p53 can cooperate with redox changes to affect cellular metabolism and survival involving intricate control of intracellular ROS levels, resulting in diverse outcomes. In fact, the sensitivity of LSCs to agents that target p53 and apoptosis of LSCs. In addition, treatment of blood samples from human patients with AML and blast-crisis CML with lipid extracts (LE) from selenium-supplemented macrophages induced apoptosis in CD34+CD38−CD123+ LSCs, which was blocked by NSAIDs. The proapoptotic effects of selenium were, in part, related to exacerbated oxidative stress in LSCs that involved NADPH oxidases, particularly Nox1. In contrast, selenium treatment did not affect normal HSCs, suggesting that LSCs are uniquely sensitive to changes in intracellular ROS. These studies suggest a new mechanism that underlies the selenium-dependent effects on the viability of LSCs, which may open new opportunities for leukemia therapy.

## Materials and Methods

### Differential selenium status in mice

Three-week-old male BALB/c mice (*n* = 7/group) for polycythemic Friend virus (FVP) infection or C57BL/6 mice (for BCR–ABL) LSC transplantation; *n* = 5–7/group) were weaned on semipurified diets (AIN76A; Harlan Teklad) that were either selenium-deficient (*<0.01 ppm Se; Se-D*), selenium adequate (*0.08 ppm Se as selenite; Se-A*), or selenium supplemented (*0.4 ppm Se as selenite; Se-S*) for 8 weeks as described earlier (9). In the FVP infection model, selenium was also used in the form of GPX1 as a surrogate marker to control of intracellular ROS levels, resulting in diverse outcomes. In fact, the sensitivity of LSCs to agents that target p53 and apoptosis of LSCs. In addition, treatment of blood samples from human patients with AML and blast-crisis CML with lipid extracts (LE) from selenium-supplemented macrophages induced apoptosis in CD34+CD38−CD123+ LSCs, which was blocked by NSAIDs. The proapoptotic effects of selenium were, in part, related to exacerbated oxidative stress in LSCs that involved NADPH oxidases, particularly Nox1. In contrast, selenium treatment did not affect normal HSCs, establishing complex regulatory networks involving temporal-segregated responses, p53 can cooperate with redox changes (29). Thus, by establishing complex regulatory networks involving temporally segregated responses, p53 can cooperate with redox changes to affect cellular metabolism and survival involving intricate control of intracellular ROS levels, resulting in diverse outcomes. In fact, the sensitivity of LSCs to agents that target p53 and apoptosis of LSCs. In addition, treatment of blood samples from human patients with AML and blast-crisis CML with lipid extracts (LE) from selenium-supplemented macrophages induced apoptosis in CD34+CD38−CD123+ LSCs, which was blocked by NSAIDs. The proapoptotic effects of selenium were, in part, related to exacerbated oxidative stress in LSCs that involved NADPH oxidases, particularly Nox1. In contrast, selenium treatment did not affect normal HSCs, suggesting that LSCs are uniquely sensitive to changes in intracellular ROS. These studies suggest a new mechanism that underlies the selenium-dependent effects on the viability of LSCs, which may open new opportunities for leukemia therapy.

### Induction of Friend erythroleukemia in mice

After completion of the diet-feeding schedule, Balb/c mice were injected with FVP via the retroorbital sinus. On day 15 after infection, splenomegaly and changes in the hematologic parameters were assessed as described earlier (32).

### Expression of BCR–ABL fusion protein in HSC and induction of CML in mice

Retroviral stocks were generated by transfecting HEK293T cells with MIGR–BCR–ABL–IRESGFP or MIGR–IRESGFP murine stem cell virus retroviral expression system (MSCV) empty plasmid (kind gift from Dr. Warren Pear, University of Pennsylvania, Philadelphia, PA) using Fugene 6 transfection reagent (Roche). Isolation and transduction of HSCs with these viruses were performed as described earlier (3, 6). LSCs (GFP+Kit+Scal1+Lin−) were isolated from spleen and bone marrow using FACS (BD Influx Cell Sorter) as described earlier (3, 6).

### Serial transplantation assays

BCR–ABL+ LSCs isolated from the bone marrow of a C57BL/6 CD45.1 donor (as described above) were transplanted into Se-A mice on a CD45.2 background. The LSCs (CD45.1+) isolated from the bone marrow of primary transplants were used for secondary transplants in CD45.2+ mice maintained on Se-A or Se-S diets. The LSCs sorted from the bone marrow (using flow cytometry) from Se-A or total bone marrow (unsorted) in the case of Se-S mice were used in a tertiary transplant into CD45.2+ Se-A mice. Leukocytosis and the presence of LSCs in the bone marrow and spleen were examined using flow cytometry.

### Histology and TUNEL staining

Formalin-fixed spleens were paraffin-embedded, sectioned, and used for hematoxylin and eosin or terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining to examine gross anatomical changes or apoptosis, respectively, following induction of leukemia as described in the Supplementary Methods.

### Gene expression analyses

Quantitative RT-PCR (qPCR) with TaqMan probes (from Life Technologies) was performed to examine the gene expression in sorted BCR–ABL+ LSCs and HSCs as described in the Supplementary Methods. Data were analyzed according to the method of Livak and Schmittgen (33) with normalization to 18S rRNA.

### Inhibition of COX-dependent ARA metabolism

Balb/c or C57BL/6 mice were administered indomethacin (0.00325% w/v), a nonselective COX inhibitor, in drinking water for 2 weeks before FVP infection or LSC (BCR–ABL) HSC transplantation, respectively, and continued for two additional weeks. Apoptosis of LSCs in the spleen and bone marrow was examined by flow cytometry. For *ex vivo* experiments, splenocytes containing LSCs or MSCV–transplanted Se-D mice were cultured in defined Iscove’s modified Dulbecco’s medium (IMDM; with a basal level of 7 nmol/L selenium) with
increasing amounts of selenium (as selenite; 0–500 nmol/L) in the presence or absence of DMSO, indomethacin (20 μmol/L; a general COX inhibitor), HQL-79 (25 μmol/L; H-PGDS inhibitor), CAI10526 (25 μmol/L; mPGES-1 inhibitor) for 36 hours. Apoptosis of LSCs and HSCs were examined using flow cytometry. To examine the effect of selenium-dependent eicosanoid switching in macrophages on the viability of LSCs, total lipid extracts (LE) of the culture media from RAW264.7 murine macrophages (1 × 10^6 cells) treated with inhibitors, including NSAIDs (ibuprofen, 20 μmol/L; naproxen, 50 μmol/L; or aspirin, 2.5 mmol/L) in the presence or absence of selenium (selenite; 250–500 nmol/L; 3 days), were isolated as described earlier (6, 9, 10). LE was added to GFP KIt "Sca1 "Lin" sorted murine CML LSCs, or to peripheral blood samples from a patient with AML as well as a patient with blast-crisis CML for 6 hours. Apoptosis of murine CML and human LSCs (AML and CML patient-derived; CD34+CD38−CD123−) was examined using Annexin V staining by flow cytometry. Human samples were obtained from the University of Rochester (Rochester, NY). All procedures were preapproved by the IBC and Institutional Review Board at University of Rochester. Western immunoblotting was performed with sorted murine CML LSCs or FVP spleen before and after treatments as described in the Supplementary Methods.

Detection of oxidative stress in LSCs and HSCs

CD34+ KIt "Sca-1 "Lin" GFP+ LSCs and CD34+ KIt "Sca-1 "Lin" GFP− HSCs were isolated from the spleen of LSC- or HSC-transplanted mice (primary donors) as described earlier. A total of 5 × 10^6 cells were plated in 1 mL of media containing IMDM supplemented with 15% FBS (ATCC; final selenium concentration of 7 nmol/L) containing 1% v/v BSA (Sigma), insulin (Sigma: 10 μg/mL), transferrin (Sigma: 200 μg/mL), L-glutamine (Cellgro; 2 mmol/L), SHH (Peprotech; 50 ng/mL), SCF (Peprotech; 50 ng/mL), GDF15 (Peprotech; 30 ng/mL), and recombinant murine IL3 (Peprotech; 10 ng/mL). Following incubation with compounds for 24 hours at 37°C, 1 μL of CellROX Deep Red Reagent (Invitrogen) was added to LSCs in 1 mL media to a final concentration of 50 nmol/L CellROX reagent and incubated for 1 hour at 37°C. Cells were washed and resuspended in Dulbecco’s Phosphate-Buffered Saline containing 2% FBS, 1% penicillin-streptomycin (Cellgro). Oxidized CellROX was analyzed on a BD Accuri C6 flow cytometer in FL-4 channel.

Effect of NADPH oxidase inhibitors and antioxidants on LSCs

Sorted CD34+ KIt "Sca1 "Lin" (GFP+) cells were treated with selenium (as selenite; 250 nmol/L) in the presence of NADPH oxidase-1 inhibitors, DPI (diphenylidonium; 100 nmol/L) or ML-171 (2-acetylphenothiazine; 1 μmol/L) for 24 hours. Similarly, LSCs were treated with NAc (1 mmol/L) or ebselen (2 mmol/L) for 24 hours and the apoptosis of GFP− cells was estimated by flow cytometry with Annexin V-positive staining. DPI, ML-171, and ebselen were prepared in DMSO. Final concentration of DMSO was 0.1% v/v. All data shown were corrected for vehicle-dependent effects that were minimal.

Statistical analyses

The results are expressed as biologic mean ± SEM and the differences between groups were analyzed using the Student t test or one-way ANOVA followed by the Tukey post-hoc test using GraphPad Prism for comparisons between various groups. The criterion for statistical significance was P < 0.05.

Results

Selenium supplementation alleviates splenomegaly and development of leukemia

We investigated whether increased levels of selenium in the diet affected the development of leukemia in a CML model. In Se-A recipients, transplantation of BCR–ABL+ LSCs led to splenomegaly and leukocytosis in the peripheral blood that is characteristic of leukemia (Fig. 1A and B). The CML mice on Se-D or Se-A diets did not survive beyond 2 to 3 weeks after transplantation compared with MSCV-GFP+ HSC–transplanted mice (data not shown). In contrast, transplantation of BCR–ABL+ LSCs into mice maintained on a Se-S diet failed to cause splenomegaly or leukocytosis (Fig. 1A and B). Analysis of bone marrow and spleen showed that the Se-S group lacked detectable LSCs (GFP+ KIt "Sca1 "Lin"; Fig. 1C), which was consistent with the lack of splenomegaly and leukocytosis (Fig. 1A and B). Similar results were also obtained with the FVP infection model, in which mice on the Se-S diet were resistant to FVP-induced erythroleukemia, whereas those on Se-D and Se-A diets succumbed to the disease (Supplementary Fig. S1). Changes in peripheral blood values of FVP-infected animals such as increased hematocrit (data not shown), leukocytes, reticulocytes, and decreased platelet levels in Se-D and Se-A diet were consistent with the onset of leukemia (Supplementary Fig. S1). Complete blood count analysis performed in the FVP-infected mice on Se-S diet exhibited significantly reduced WBCs and reticulocytes when compared with FVP-infected Se-D and Se-A mice. This effect was observed with selenium supplementation in the form of both inorganic selenium and organic (and bioavailable) MSA. Flow cytometric analysis indicated that selenium supplementation with selenium (0.4 ppm) or MSA (3 ppm) significantly ablated FVP–LSCs in the spleen (Supplementary Fig. S1) and bone marrow (data not shown). Furthermore, histologic examination of the splenic sections suggested that selenium supplementation also resulted in a complete return to normal splenic histioarchitecture in the FVP-infected group (Supplementary Fig. S2). Together, these results support the notion that supraphysiologic levels of selenium specifically affect the proliferation of LSCs, but not HSCs.

Supplementation of mice with selenium increases apoptosis of LSCs

To demonstrate supranutritional levels of selenium in the diet led to apoptosis of LSCs, we examined splenic sections from the three diet groups transplanted with LSCs using TUNEL assays. Increased TUNEL-positive staining in Se-S mice transplanted with BCR–ABL+ LSC was seen when compared with Se-D or Se-A groups (Fig. 1D). TUNEL staining of the MSCV-GFP+ –transplanted HSC controls did not show any changes between the diet groups (data not shown). Increased
TUNEL staining of the splenic sections from FVP-infected Se-S mice was also seen when compared with the FVP-infected mice fed Se-D or Se-A diets (Supplementary Fig. S3). In addition, we examined the sensitivity of LSCs from Se-D spleen in an ex vivo analysis of apoptosis in response to exogenous selenium at concentrations that reflect in vivo diet-derived levels. Inorganic selenium (as sodium selenite) was added at concentrations varying from 0 to 500 nmol/L to total splenocytes isolated from Se-D mice transplanted with BCR–ABL+ LSCs or MSCV-GFP+ HSCs. A dose-dependent decrease in GFP+ cells was seen in the BCR–ABL+ LSC-transplanted mice on Se-D, Se-A, or Se-S diets; untransplanted Se-S splenic section used as a negative control. Representative images with TUNEL-positive staining are shown. Total area of TUNEL+ staining is shown below each panel.

Serial transplantation of LSCs from Se-S mice fails to cause leukemia

Serial transplantation assays were used to further demonstrate that Se-S mice did not contain residual LSCs. In this assay, we generated LSCs using congenic CD45.1 mice (GFP+ Kit+ Sca1+ Lin-). These LSCs from CD45.1 mice were transplanted into Se-A or Se-S CD45.2 mice and the progression of the disease was followed (Fig. 3A). Only the secondary transplants into Se-A recipients led to the development of the disease, whereas bone marrow transplants from Se-A donors into Se-S recipients failed to cause leukemia. Flow cytometric analysis of the spleen in these secondary recipients showed total absence of LSCs only in the Se-S group (Fig. 3B). Splenomegaly and leukocytosis was observed only in the Se-A recipients, but not in the Se-S group (Fig. 3C and D). LSCs in the bone marrow of secondary transplant Se-S recipients could not be detected, which is in contrast with those recipients that were on Se-A diets (Fig. 3E). Furthermore, we performed tertiary bone marrow transplants into Se-A recipients, in which complete bone marrow was used. As expected, transplantation from Se-A (secondary) donors led to the disease, as seen in the form of LSCs in the bone marrow and spleen (Fig. 3F), whereas total bone marrow from Se-S donor (secondary) failed to cause leukemia in tertiary Se-A recipients (Fig. 3G and H). These studies suggest that supraphysiologic levels (0.4 ppm) of selenium limit the survival of LSCs.

Endogenous CyPGs mediate the selenium-dependent eradication of LSC

Treatment of splenocytes from BCR–ABL+ LSC–transplanted Se-D mice with selenite ablated the GFP+...
showed a substantial number of GFP-cytometric analysis of the bone marrow of these mice (WBC), lymphocytes, and neutrophils (Fig. 4C and D). Flow
selenium, resulting in increased white blood cell count indomethacin-treated Se-S mice blocked the protective role of
ABL–observed in the BCR
spleen (data not shown). Similarly, the antileukemic effects signi
an increase in M34
WBC, and neutrophils in the peripheral blood along with mice infected with FVP exhibited increased hematocrit, Se-S group infected with FVP. Indomethacin-treated Se-S mice infected with FVP exhibited increased hematocrit, WBC, and neutrophils in the peripheral blood along with an increase in M34"Kit+"Sca1+"Lin" LSCs (Supplementary Fig. S4). Finally, intraperitoneal administration by 15d-PGJ2 (at 0.025 mg/kg/d for 1 week) to the indomethacin-treated FVP-infected Se-S mice rescued these mice and led to a significant decrease in LSCs in the bone marrow, whereas treatment of leukemic FVP-infected mice with 16,16-dimethyl-PGE2 (at 0.025 mg/kg/d for 1 week) had no effect on the viability of LSCs (Supplementary Fig. S4). These data demonstrate a key role for the COX–PGDS axis in the proapoptotic effects of selenium in LSCs.

**LEs from selenium-treated macrophages induce apoptosis in CML and AML patient samples**

We next tested whether selenium treatment of macrophages could induce the production of lipid mediators that would affect LSCs in human blast-crisis CML and AML patient-derived samples. Only the LE isolated from macrophages cultured in selenium-supplemented media led to significant apoptosis of CD34"CD38"CD123" LSCs. In contrast, LE isolated from HQL-79–treated or NSAID-treated RAW 264.7 macrophages failed to induce apoptosis of LSCs in both AML (Fig. 4F) and blast-crisis CML samples (Fig. 4G). Furthermore, treatment of human AML and blast-crisis CML samples with Δ12-PGJ2 (100 nmol/L) significantly reduced their colony-forming ability in methylcellulose media (data not shown). Taken together, these findings demonstrate that selenium-dependent shunting of the ARA pathway, leading to the production of CyPgs, effectively targets murine and human LSCs for apoptosis.

**Selenium induces oxidative stress in LSCs**

ROS production was examined in sorted BCR–ABL+ LSCs and MSCV-GFP+ HSCs upon treatment with selenium or LEs from selenium-treated macrophages. When compared with HSCs, BCR–ABL+ LSCs expressed lower levels of ROS (Fig. 5A). Addition of selenium in the form of selenite (250–500 nmol/L) or LEs from selenium-treated macrophages significantly increased ROS in LSCs. In contrast, cotreatment of selenium (500 nmol/L) with NSAIDs (indomethacin and aspirin) or LEs from NSAID (indomethacin, aspirin, ibuprofen, or naproxen)-treated selenium-supplemented (500 nmol/L) macrophages decreased ROS in the LSCs (Fig. 5B). Interestingly, addition of increasing amounts of selenium failed to increase ROS in HSCs and cotreatment of selenium (500 nmol/L) with NSAIDs had little effect on ROS levels in HSCs (Fig. 5C). Treatment of BCR–ABL+ LSCs with Δ12-PGJ2 or 15d-PGJ2 (at 25 nmol/L) increased intracellular ROS in LSCs to levels that were seen in LSCs treated with H2O2 or daunorubicin (Fig. 5D). LSCs treated with selenium showed a significant increase in the expression of NADPH oxidase Nox1, but not Nox2 and Nox3 was decreased (Supplementary Fig. S5). Treatment of BCR–ABL+ LSCs with NAc, an antioxidant prodrug, or NOX inhibitors such as DPI or ML171, blocked the
proapoptotic property of selenium (Fig. 5E). Increased levels of ROS in LSCs were accompanied by dose-dependent increase in the expression of two important antioxidant genes, *Sod2* and *Cat*, whereas a similar treatment failed to increase such an antioxidant response in HSCs (Fig. 5F). A similar trend was also observed in LSCs from FVP-infected spleen in which the expression of *Cat* and *Sod2* was upregulated in the Se-S diet fed groups (Supplementary Fig. S6). BCR–ABL<sup>+</sup> LSCs treated with selenium exhibited increased expression of phospholipid glutathione peroxidase (*Gpx4*; Supplementary Fig. S5). Treatment of BCR–ABL<sup>+</sup> LSCs with ebselen, a glutathione peroxidase mimetic, abrogated the selenium-mediated apoptosis (Fig. 5E). Indomethacin treatment of Se-S BCR–ABL<sup>+</sup> LSCs or LEs from indomethacin-treated macrophages cultured with selenium abrogated the increase in *Sod2* and *Cat*. In addition to these antioxidant genes, we analyzed the expression of a few p53 target genes, particularly those that regulate glycolysis and OXPHOS, in LSCs and HSCs following treatment with selenium.

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**Figure 3.** Selenium status of mice is a critical regulator of LSC viability as seen by serial transplantation experiments. A, schematic illustration of serial transplantation of BCR–ABL<sup>+</sup> LSCs. B, flow cytometric analysis of splenic LSCs (GFP<sup>+</sup>*Kit<sup>+</sup>*Sca1<sup>+</sup>*Lin<sup>-</sup>/C<sup>0</sup>) from the secondary transplant groups. C, splenomegaly. D, WBC counts on days 16 and 32 after transplantation. Range of normal levels of WBC in mice is indicated by dotted lines; a, *P* < 0.05 compared with Se-A on day 16; b, *P* < 0.05 compared with Se-S on day 16; c, *P* < 0.05 compared with Se-S day 32. E, GFP<sup>+</sup> cells in the bone marrow of secondary transplant recipients. F, flow cytometric analysis of spleen and bone marrow for the presence of LSCs in tertiary transplant groups. G, splenomegaly. H, WBC in peripheral blood of tertiary transplant recipients. All data shown are mean ± SEM and representative of n = 5 in each group of donor and recipient mice; *, *P* < 0.05.
As shown in Fig. 5G, the expression of p21, Sco2, Tigar, and Rm2b was significantly increased in LSCs treated with selenite when compared with untreated LSCs or HSCs cultured with or without selenite treatment for 6 hours ex vivo. On the other hand, expression of Sesn1, Sesn2, and Gls2 was decreased in LSCs when compared with HSCs and treatment of LSCs with selenite failed to increase their expression. These studies suggest that selenium exacerbates the production of intracellular ROS, in part, by suppressing pathways that offer antioxidant protection while activating mitochondrial OXPHOS-dependent ROS production that subsequently leads to apoptosis of LSCs.
The p53 pathway is activated by selenium in LSCs

On the basis of the increased activation of p21 and increased oxidative stress–dependent DNA damage by selenium in primary LSCs (Fig. 5G) and other cell lines (23), we examined the activation of the ATM–p53 axis in LSCs. Irrespective of the leukemia model used, mice on Se-S diet exhibited a significant
increase in the expression of p53 when compared with the Se-D group (Fig. 6A; Supplementary Fig. S7A). BCR–ABL GFP⁺ LSCs treated with either LEs from selenium-treated macrophage media or cultured directly in the presence of selenium (250–500 nmol/L) led to increased expression of p53 transcript levels, which was effectively abrogated by indomethacin. 

Figure 6. Activation of the ATM–p53 axis is critical for apoptosis in LSCs. A, Western immunoblot analysis showing p53 expression in splenocytes from BCR–ABL⁺ LSC– and MSCV-HSC–transplanted mice. GPX1 expression was used to confirm selenium status. B, expression of p53 in sorted LSCs and HSCs upon treatment with exogenous selenium (as selenite; solid gray line) or LEs (dotted line) in the presence or absence of indomethacin (INDO). Representative of n = 3 to 4 independent experiments. C, effect of other NSAIDs on selenium-induced p53 expression as seen by semiquantitative PCR. Lanes 1 to 8 represent untreated, 250 nmol/L selenite, 500 nmol/L selenite (as selenite), LE from macrophages (500 nmol/L selenite), LE from 500 nmol/L selenite-treated macrophages with indomethacin, HQL-79, naproxen, and ibuprofen, respectively. D, ATM inhibitor (KU55933; 10 nmol/L) blocks the proapoptotic effect of selenite (250 nmol/L) in BCR–ABL LSCs. Live GFP⁺ cells following treatment with DMSO (vehicle) or KU55933 are shown in selenite-treated LSCs for 24 hours. Mean ± SEM of n = 3 independent experiment; *P < 0.05. E, densitometric analysis of immunoblotting data demonstrating the activation of the Chk2–p53 axis in BCR–ABL⁺ LSCs treated with selenite (0–500 nmol/L) for 6 hours. All data shown are mean ± SEM of n = 5; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001, respectively. F, activation of p53 in peripheral blood sample of patient with AML upon treatment with selenium in the presence or absence of NSAIDs. All NSAID treatments were performed on LSCs in the presence of selenite (500 nmol/L selenium); mean ± SEM *, P < 0.05.
Selenium Targets Leukemia Stem Cells

In contrast with the response in LSCs, treatment of HSCs with selenium or LEs had no effect on p53 expression (Fig. 6B). Furthermore, pretreatment of splenocytes from BCR–ABL GFP	supplemented mice with an ATM kinase inhibitor (KU55933; 10 nmol/L) followed by treatment with selenite (250 nmol/L) blocked the proapoptotic effect of selenium (Fig. 6D). As a consequence of ATM kinase activation, phosphorylated Chk-2 and P-p53 (Ser15) were increased in sorted BCR–ABL+ LSCs supplemented with selenium (100 and 500 nmol/L; Fig. 6E). A similar trend was also observed in the splenocytes of Se-S and Se-MSA fed groups infected with FVP (Supplementary Fig. S7B). Increase in the levels of activated caspase-3 and subsequent cleavage of PARP was seen in the selenite-treated LSCs. Treatment of AML patient cells with LEs from selenium-treated macrophages upregulated p53 expression, whereas those from NSAID-treated macrophages blocked the effect of selenium (Fig. 6F). These results suggest that selenium-dependent metabolic shunting of the ARA pathway sensitizes murine CML and human CML and AML LSCs for apoptosis via the activation of the ATM–p53 axis.

Discussion

The anticarcinogenic activity of selenium has been demonstrated in many cancer cell lines. However, there are no reports that specifically focus on the targeting of CSCs by selenium at concentrations that are achievable through dietary supplementation without any apparent toxic effects. Here, we demonstrate a novel mechanism in which selenium supplementation, at supraphysiologic and nontoxic doses, enhances the production of endogenous PGD2-derived CyPGs to trigger selective apoptosis of LSCs by increasing intracellular oxidative stress.

Previous studies have suggested that BCR–ABL expression was associated with a temporally regulated increase in oxidative stress by modulating upstream signaling events that had a bearing on cell fate decisions (34–37). This increase was also associated with induction of self-mutations to encode imatinib resistance. In contrast, comparison of the baseline ROS levels associated with induction of self-mutations to encode imatinib resistance. In addition, BCR–ABL expressing HSCs used here are primary cells that differ from many hematopoietic cell lines previously used to suggest an association between ROS and BCR–ABL expression (34, 35). Regardless of the baseline differences in ROS, treatment of LSCs with selenium or LEs exacerbated oxidative stress. Although it is not clear how endogenous CyPGs increase ROS, exogenous addition of high concentrations (μmol/L) of 15d-PGJ2 has been shown to increase intracellular iron, possibly as a bystander effect of electrophilic stress, to exacerbate ROS in CG3 human thyroid papillary cancer cells (38). This angle is worthy of further investigation to examine whether a similar mechanism holds good even with low endogenes levels of CyPGs and/or increased cellular selenium. Needless to say, the mechanisms by which selenium or endogenous CyPGs activate the ATM–p53 axis of apoptosis in LSCs are currently being investigated.

Our studies suggest that selenium in the form of selenoproteins and/or metabolites of selenium may play a critical role in LSC apoptosis. Increased LSC expression of Sod2, Cat, and Gpx4 to compensate for increased ROS corroborates well with the increased expression of Nox1 in LSCs treated with supra-physiologic doses of selenium as reported earlier in other cancer cells (39). In addition, the ability of two NOX inhibitors, DPI and ML171, and glutathione precursor prodrug NAc and ebselen to inhibit selenium-dependent apoptosis further suggests that LSCs are endowed with a suboptimal antioxidant response system that favors apoptosis. It also seems that excess selenium overrides these antioxidant defenses, leading to increased ROS-dependent genomic instability and apoptosis through an unknown mechanism. This idea is consistent with the decrease in the expression of Sesn1, Sesn2, and Gls2 coupled to an increase in the expression of Sco2, Tigar, and Rm2b (P53r2). These observations point to the ability of selenium to favor a decrease in aerobic glycolysis in LSCs through the increased expression of Tigar, which may effectively inhibit glycolysis to activate NADPH production through the pentose phosphate pathway. Increased oxygen consumption and mitochondrial respiration as a consequence of upregulated Sco2 and Rm2b could also contribute to higher levels of ROS to effectively reverse the Warburg effect to favor apoptosis.

Our studies showed that organic forms of selenium, such as Se-Met and pXSC, were not as effective as MSA in LSC apoptosis. Inorganic selenium was more effective than MSA in vivo (FVP model) and in ex vivo assays with CML LSCs. This is in contrast with a mammary hyperplastic epithelial tumor model in which MSA was shown to be more efficacious (31). Such model-specific differences in sensitivity could be due to the inability of these compounds to skew pathways of ARA metabolism to the same extent as selenite (9).

Although our data presented here implicate macrophages as one of the key producers of PGD2–derived CyPGs, the likelihood of LSCs also contributing endogenous Δ12-PGJ2 (and 15d-PGJ2) in a selenoprotein-dependent manner cannot be ruled out. In fact, increased production of Δ12-PGJ2 was accompanied by an increase in the expression of Hpgds and prominent selenoproteins, Gpx1, Gpx4, and Txnrd1 in LSCs (Supplementary Fig. S8), which is in agreement with our previous reports in macrophages (9). Although much smaller in magnitude, Δ12-PGJ2 produced at approximately 6 (nmol/L)/1 × 106 LSCs in response to selenium (500 nmol/L) still raises the possibility of an autocrine mechanism of apoptosis of BCR–ABL+ LSCs and FVP-LSCs (6). However, with the possibility of LSCs being outnumbered by monocytes, macrophages, and T cells, which express COX-1/2 and H-PGDS (5, 40, 41) in the spleen and bone marrow microenvironment, the role of the immune component as CyPG producers in response to selenium cannot be ignored. Regardless of the origin of CyPGs, the ability of...
selenoproteins to increase sensitivity of only the LSCs to endogenous CyPGs is intriguing and needs to be probed further.

The abrogation of increased sensitivity of LSCs to selenium with NSAIDs and HQL-79 suggests that the endogenous prostanooids, specifically PGD$_2$-derived CyPGs, mediate the selective eradication of LSCs. Interestingly, the role of COX in leukemia has been implicated in epidemiologic studies. An increased risk of adult leukemia was associated with the use of aspirin and non-aspirin NSAIDs (7, 8). Hart reported that indomethacin increased mortality of mice bearing BCL1, leukemia (42). Thus, our studies suggest a confounding role for NSAIDs in clinical trials, particularly those involving supraphysiologic levels of selenium as in the Selenium and Vitamin E Cancer Prevention Trial for prostate cancer (43, 44), in which NSAID users were not excluded from the study. In conclusion, we report the ability of selenium to selectively eradicate LSCs in two well-studied murine models of leukemia and also in AML and blast-crisis CML patient samples. Our data support endogenous eicosanoids, such as CyPGs, to be critical in the proapoptotic function of selenium. The selectivity of endogenous CyPGs to target the LSCs for apoptosis highlights the importance of redox-dependent pathways as major determinants of LSC viability. Although these studies suggest the use of selenium supplementation as an adjunct therapy for CML, the success of this therapy will ultimately rest on the efficient expression of selenoproteins and their ability to skew ARA metabolism to upregulate CyPG production.

### References


### Disclosure of Potential Conflicts of Interest

R.F. Paulson is president of, and has ownership interest (including patents) in, OncOmega Inc. K.S. Prabhu is a scientific cofounder and has ownership interest (including patents) in OncOmega Inc. No potential conflicts of interest were disclosed.

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Selenium Targets Leukemia Stem Cells

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