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THE ROLE OF SELENIUM IN LEUKEMIA STEM CELL APOPTOSIS

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Abstract

Selenium is an essential micronutrient that has important anti-inflammatory and anti-carcinogenic properties. Inflammatory stimuli upregulate the expression of cyclooxygenase-2 (COX-2), which increases the synthesis of inflammatory prostaglandins such as PGE2 as well as anti-inflammatory PGD_2. The J-series prostaglandins, downstream metabolites of PGD_2 have been shown to have anti-inflammatory and anti-tumorigenic properties, especially 15d-PGJ_2. 15d-PGJ_2 functions through multiple mechanisms, peroxisome proliferator activated receptor (PPAR)γ-dependent and independent, to induce apoptosis in cancerous cells, including Friend virus derived LSCs.

We proposed that the anti-carcinogenic effects of Se are due to its upregulation of the terminal prostaglandin 15d-PGJ_2. In order to test this hypothesis, a biologic assay for the production of 15d-PGJ_2 in Se-supplemented cells was created. LPS-stimulated RAW 246.7 murine macrophages were grown under Se-supplemented or deficient conditions and treated with inhibitors of prostaglandin synthesis originating from AA metabolism, such as indomethacin, HQL-79, and CAY10526. Lipid extracts from these cells used to treat FV-LSCs induced apoptosis only in those samples that were Se-supplemented and endogenous production of 15d-PGJ_2 was not inhibited, while the inhibition of PGE_2 synthesis had no effect.

Friend virus is a murine model of AML. The relapse rate for AML patients exceeds 80% because LSCs are highly resistant to conventional chemotherapy protocols. 15d-PGJ_2 selectively induces apoptosis in LSCs. These preliminary studies suggest that upregulating 15d-PGJ_2 synthesis by supplementing AML patients with Se is a promising therapeutic endpoint that is worth further investigation to pursue the potential chemotherapeutic and chemopreventive potential of Se.

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Literature Review

Inflammation

Inflammation is an adaptive response by an organism to remove injurious stimuli and promote healing of damaged tissue. It is a complex response regulated by cellular mediators produced by cells of the immune system. Pro-inflammatory mediators are responsible for propagating acute inflammation. Alteration of inflammatory signals in favor of anti-inflammatory mediators leads to resolution of inflammation (Parham, 2009).

Tissue damage resulting from the original insult or from the inflammatory response may prevent resolution. Irreversibly damaged cells die by necrosis or apoptosis. Oxidative stress is a major inducer of apoptosis in cells. Oxidative stress occurs when reactive oxygen species (ROS) accumulate in a cell due to increased production or an exhaustion of cellular antioxidant systems (El-Bayoumy, 2001). ROS cause damage to DNA, lipid peroxidation, and oxidation of proteins. ROS are also a by-product of the electron transport chain in mitochondria and are quickly detoxified by cellular systems. Large amounts of ROS are produced by the respiratory burst in activated macrophages (Forman and Torres, 2002). ROS are responsible for the antimicrobial action of these cells.

Eukaryotic cells have evolved multiple pathways to neutralize ROS and prevent oxidative damage. Individual enzymes include superoxide dismutase and catalase. Additionally, there are two important antioxidant systems: thioredoxin and glutathione. Thioredoxin is an effective reducing agent due to the presence of two nucleophilic cysteine residues. Thioredoxin is maintained in its reduced state by the selenoprotein thioredoxin reductase (TrxR), which uses NADPH as an electron donor (Lu and Holmgren, 2009). Glutathione (GSH) is a tripeptide synthesized from the amino acids cysteine, glycine, and glutamate. The thiol group in GSH acts as an electron donor, thus GSH is a reducing agent. The oxidized form of GSH, glutathione disulfide (GSSG), is reduced by glutathione reductase using FADH- anion (Lu and Holmgren,

2009). Glutathione is also an essential cofactor for the selenoprotein, gluthatione peroxidases (GPXs). GPXs protect cells by reducing lipid hyperperoxides and hydrogen peroxide to respective alcohols and water.

Prostaglandin Synthesis

Prostaglandins are an important class of mediators derived from polyunsaturated fatty acids. All prostaglandins are carboxylic acids containing a cyclopentane ring. There are three series of prostaglandins classified by their respective precursor molecules; gamma linoleic acid, arachidonic acid, and eicosapentaenoic acid. Series-2 derived from arachidonic acid (AA), is the predominant class (see Figure 1). Phospholipase A_2 cleaves arachidonate from membrane phospholipids providing a substrate for cyclooxygenase (COX). COX converts arachidonate to PGG_2 which is then further reduced to PGH_2. There are two isoforms of COX that are expressed in mammalian cells. While COX 1 is constitutively active, COX 2 expression is induced by inflammatory stimuli (Surh et al., 2011).

PGH_2 is the central prostanoid precursor, metabolized to PGD_2, PGE_2, PGI_2, and thromboxane A_2 (TXA_2). Specific inducible enzymes are responsible for the conversion of PGH_2 to each terminal prostanoid (Surh et al., 2011). PGE_2 is a potent pro-inflammatory mediator; its conversion from PGH_2 is catalyzed by microsomal PGE synthase-1. PGD synthase (PGDS) catalyzes the production of PGD_2. There are two distinct isoforms: lipocalin-type PGDS (L-PGDS) and hematopoietic PGDS (H-PGDS). H-PGDS is the form expressed by macrophages. H-PGDS requires GSH cofactor to cleave the cyclopentane epoxy bridge in PGH_2 (Herlong and Scott, 2006).

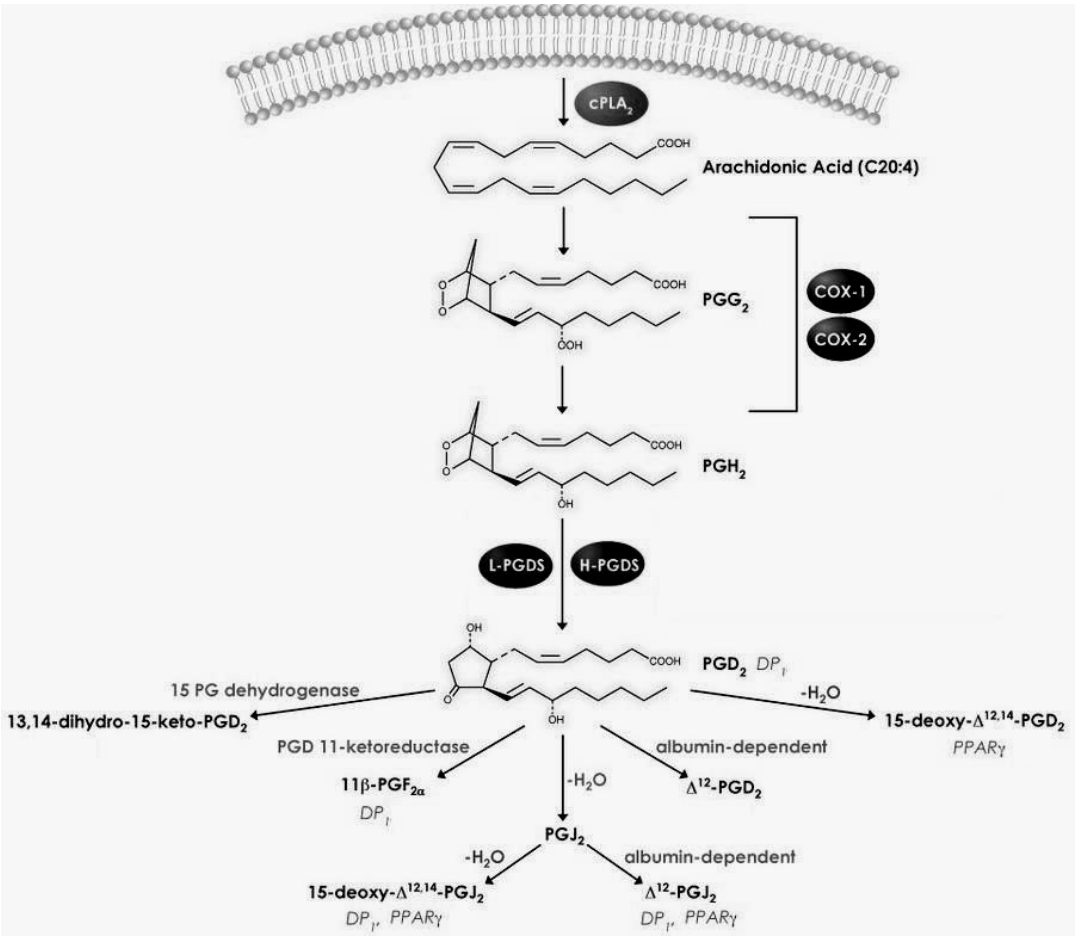

Figure 1. Prostaglandin synthesis cascade.
Adapted from: May O. 2009.

PGD_2 is readily dehydrated to PGJ_2, the precursor of the J series prostaglandins (see Figure 2). Spontaneous dehydration of PGD_2 can be enhanced by albumin-induced catalysis (Bell-Parikh et al., 2003). Rearrangement of PGJ_2 yields Δ^{12}-PGJ_2 which is further dehydrated to 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) (Kansanen et al., 2009).

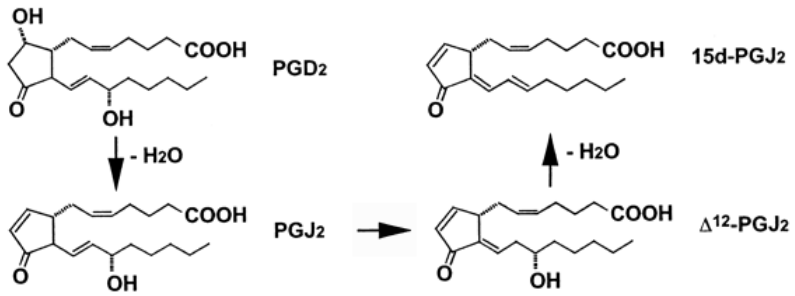

Figure 2. Structure of J series prostaglandins.
Adapted from: Kansanen et al. 2009.

Mechanism of 15-deoxy-$\Delta^{12,14}$-PGJ_2 Action

15d-PGJ_2 has potent anti-inflammatory effects. Its biologic activity is dependent on its structure. Members of the J-series possess a reactive cyclopentenone ring (Bell-Parikh et al., 2003). In 15d-PGJ_2, the ring contains a highly electrophilic α, β-unsaturated ketone. Michael addition reactions occur between the electrophilic carbonyl group and nucleophilic groups on proteins, such as cysteine thiols (Surh et al., 2011) (see Figure 3). These prostaglandins impact gene expression by forming covalent adducts with proteins, including transcription factors in the cytoplasm. Alternatively, oxidation of specific cysteine residues may initiate redox signaling cascades (Surh et al., 2011). The importance of the carbonyl group is demonstrated by the 15d-PGJ_2 analog, 9,10-dihydro-15d-PGJ_2, which lacks an unsaturation at carbon nine and also lacks the biologic activity of 15d-PGJ_2 (Hedge et al., 2011). Thus, endogenous regulation of production of such electrophilic PGs could serve as a novel mechanism of anti-inflammation, with additional benefits, in biological systems.

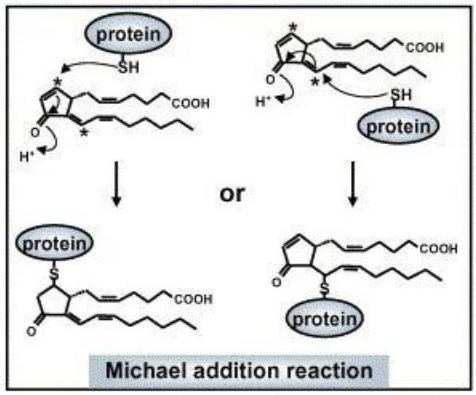

Figure 3. Michael addition reactions between the highly electrophonic carbon in 15d-PGJ_2 and nucleophilic thiol groups in proteins.
Source: Kim and Surh. 2006.

Concentrations of 15d-PGJ_2 produced *in vivo* has been notoriously difficult to quantify. The negligible levels measured by ELISA and other techniques have led to controversy regarding the biologic significance of the molecule. Controversy also arose because large

quantities (2.5 to 100 μM) of exogenous 15d-PGJ_2 were required in culture to elicit a biologic effect. The reactivity of 15d-PGJ_2 with numerous biomolecules *in vitro* accounts for this discrepancy (Bell-Parikh et al., 2003). 50 to 80% of 15d-PGJ_2 added to culture is bound in the serum (Rajakariar et al., 2007). Elsewhere it has been reported that only 1 to 3% of exogenous 15d-PGJ_2 is available for cellular signaling (Kansanen et al., 2009). Rajakariar et al. (2007) succeeded in quantifying 15d-PGJ_2 using liquid chromatography-mass spectrometry (LC-MS). They found levels of 15d-PGJ_2 ranging from 0.5 to 5 ng/mL in murine inflammatory exudates during the resolution phase of inflammation.

15d-PGJ_2 exhibits potent anti-inflammatory effects by inhibiting the synthesis of inflammatory cytokines and inducible nitric oxide synthase (iNOS) in activated macrophages through a PPARγ-dependent mechanism (Ricote et al., 1998). 15d-PGJ_2 is an endogenous ligand for PPARγ, a member of the peroxisome proliferator-activated receptor family of nuclear transcription factors. PPARγ is highly expressed in adipose tissue, colon, and macrophages. PPARγ can upregulate gene expression by heterodimerizing with the retinoic acid receptor (RXR) and binding to its cognate response element. Conversely, PPARγ can downregulate gene expression through transrepression of transcription factors or sequestration of coactivators (Coyle et al., 2005). Acting through a PPARγ-dependent mechanism 15d-PGJ_2 is a negative regulator of AP-1, STAT, and NF-κB (Ricote et al., 1998).

15d-PGJ_2 can also inhibit the transcriptional activity of NF-κB in a PPARγ-independent manner (Rossi et al., 2000). 15d-PGJ_2 prevents the nuclear translocation of NF-κB by inhibiting IκB kinase (IKK) through direct modification of the cysteine residues in the β subunit. IKK is responsible for the phosphorylation and subsequent degradation of the NF-κB repressor IκBα. Without IKK activity NF-κB remains sequestered in the cytoplasm. COX-2 is one of the many enzymes which expression is up-regulated by NF-κB, thus inhibition of NF-κB signaling by 15d-PGJ_2 leads to the down-regulation of COX-2 mediated inflammation and contributes to

resolution (Rossi et al., 2000).

Some of the anti-inflammatory and cytoprotective effects of 15d-PGJ_2 are mediated through the binding of nuclear factor-E2-related factor 2 (Nrf2) to antioxidant response elements (ARE) (Kansanen et al., 2009). ARE sequence act as promoters for genes with antioxidant, anti-inflammatory and protective effects against oxidative and electrophilic stress. Binding of Nrf2 to the tethering protein Keap1 results in its ubiquitination and rapid degradation. Modification of cysteine residues in Keap1 by 15d-PGJ_2 or other electrophiles releases Nrf2 and promotes transcription (Kansanen et al., 2009).

15d-PGJ_2 can induce apoptosis by p53 through direct modification of ataxia-telangiectasia mutated (ATM) (Kobayashi et al., 2006). ATM is a protein kinase which plays a critical role in the regulation of cell cycle checkpoints through the phosphorylation of p53, Chk2, and c-Abl. Covalent binding of the electrophilic α,β-unsaturated ketone in 15d-PGJ_2 to redox-sensitive sulfhydryl groups in ATM activates its kinase activity. ATM activated by 15d-PGJ_2 selectively phosphorylates p53 and subsequently induces apoptosis (Kobayashi et al., 2006).

The oxidative properties of 15d-PGJ_2 induce the production of ROS and the downstream induction of apoptosis. Mitochondria and NAPDH oxidase are important sources of ROS produced in response to 15d-PGJ_2 (Shin et al., 2009). ROS mediated activation of executioner caspase 3 and subsequent cleavage of poly (ADP-ribose) polymerase (PARP) result in apoptosis in human leukemia cells (Chen et al., 2005). 15d-PGJ_2 accelerates caspase-dependent apoptosis in neutrophils and eosinophils which promotes the resolution of apoptosis (Ward et al., 2002).

15d-PGJ_2 mediated ROS formation also rapidly downregulates the expression and phosphorylation of Akt. Akt is a member of the serine/threonine kinase family and has potent anti-apoptotic and proliferative functions (Han et al., 2007). Activation of JNK in response to 15d-PGJ_2-dependent ROS production is believed to be responsible for the decrease in pAkt (Shin et al., 2009). Akt signaling confers resistance to tumor necrosis factor-related apoptosis-

inducing ligand (TRAIL) inhibiting apoptosis in leukemia cell lines. Binding of TRAIL to death receptors activates Fas-associated death domain (FADD) which in conjunction with procaspase 8 forms the death-inducing signaling complex (DISC) and initiates the executioner caspase cascade and the mitochondrial apoptotic pathway (Han et al., 2007; Shin et al., 2009).

15d-PGJ_2 can additionally activate the mitochondrial apoptotic pathway through regulation of the Bcl-2 family. 15d-PGJ_2 downregulates the expression of anti-apoptotic members, such as Bcl-2, and upregulates the expression of pro-apoptotic members, such as Bax. Bax regulates mitochondrial membrane permeability; upregulation allows the escape of cytochrome C into the cytoplasm which activates the executioner caspase cascade (Lin et al., 2006) (see Figure 4).

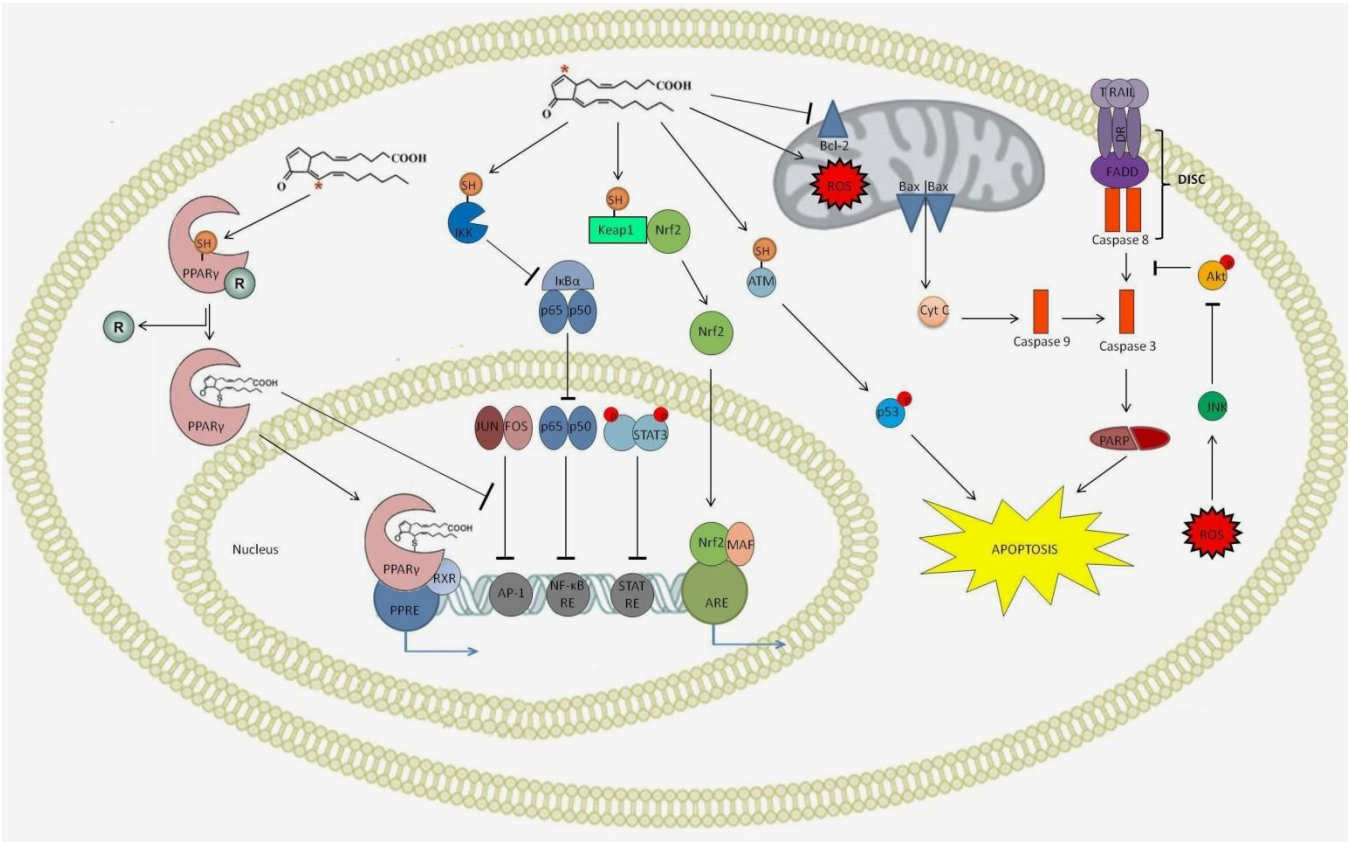

Figure 4. PPARγ-dependent and independent mechanisms responsible for the anti-inflammatory and pro-apoptotic activity of 15d-PGJ_2.

The Effects of Selenium on Inflammation

Selenium is an essential micronutrient. Selenium is provided in the diet in inorganic forms, sodium selenite (Se^{4+}) and selenate (Se^{6+}), and organic forms, selenomethionine (SeMet) and Se-methylselenocysteine (Zeng, 2009). The reduced species of selenium (selenide; Se^{2-}) released from organic molecules can be immediately utilized by the host. Oxidized species of selenium released from inorganic salts must be reduced to selenide by glutathione prior to utilization (Zeng, 2009).

Organisms incorporate selenium into proteins by two distinct pathways. In the primary pathway, selenocysteine (SeCys) synthesized *de novo* in the cell is specifically incorporated into proteins. SeCys has the same structure as cysteine except for the replacement of sulfur by selenium. Selenocysteine synthase replaces the hydroxyl group of tRNA-bound serine with a selenol moiety (-SeH) donated by monoselenophosphate ($SePO_3^{3-}$). Selenophosphate synthetase 2 (SPS2) catalyzes the conversion of selenide to monoselenophosphate (see Figure 5). SeCys-insertion sequence elements (SECIS), an untranslated region at the 3' end of selenoprotein mRNA recodes tRNA to insert SeCys at UGA sequences, normally a stop codon (Lu and Holmgren, 2009). Alternatively, SeMet may be nonspecifically incorporated into proteins in the place of methionine (Zeng, 2009).

There are over 25 known selenoproteins in humans, the best studied of which are TrxRs and GPXs. As discussed previously, TrxR and GPX maintain the reducing capacity of cellular antioxidant systems (Lu, 2009). Due to its influence on the redox state of the cell, Se is a potent regulator of transcription pathways. Oxidative stress in Se-deficient cells results in the transcription of NF-κB responsive genes (Vunta et al., 2007). Conversely, inactivation of NF-κB in Se-supplemented cells inhibits the expression of pro-inflammatory genes, such as COX-2 and nitric oxide synthase (Zamamiri-Davis et al., 2002; Prabhu et al., 2002; Vunta et al., 2007). Selenium also indirectly regulates c-Jun N-terminal kinase (JNK) and activating protein-1 (AP-

1) via the redox sensitive cysteine moieties (Zeng and Combs, 2008).

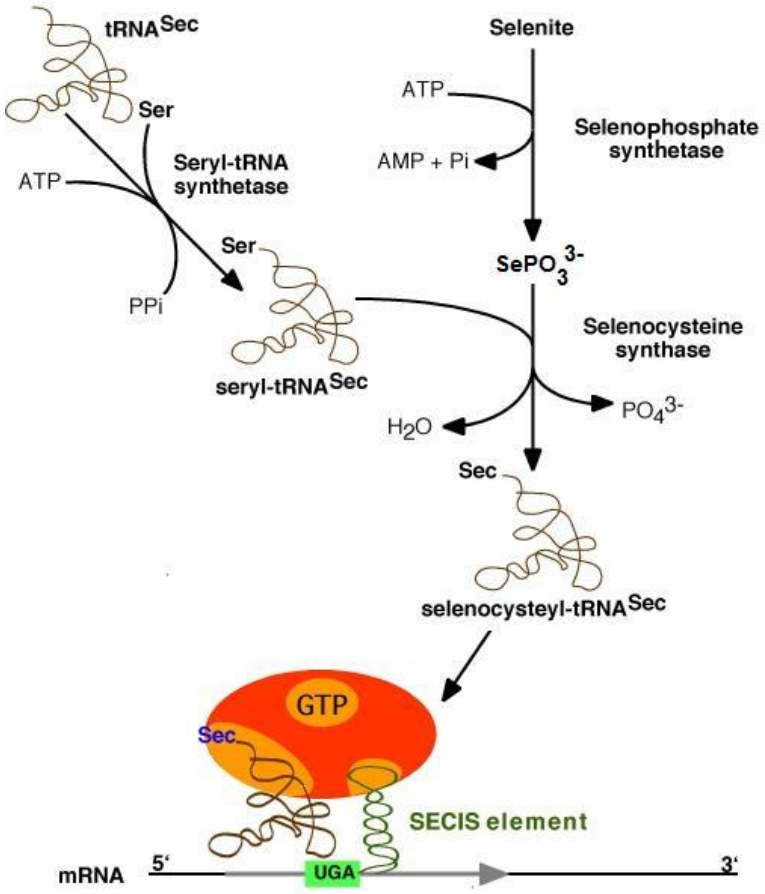

Figure 5. Synthesis of selenocysteine.
Adapted from: Stock T et al. 2011.

The biologic effects of selenium cannot be wholly attributed to its antioxidant function. Selenium has diverse impact on cell proliferation, immune surveillance, and carcinogen metabolism (Zeng, 2009). Cells cannot progress through the cell cycle checkpoints in the absence of selenium. Nanomolar concentrations of selenium are required for cell growth in culture media. Selenium upregulates the expression of cyclin C, c-Myc, and cyclin-dependent kinases as well as increasing protein phosphorylation (Zeng and Combs, 2008). Additionally, adequate selenium levels suppress the activity of apoptosis-regulating kinase 1 (ASK 1) promoting cell survival (Lu and Holmgren, 2009). Interestingly Se-supplementation has the opposite effect on cell cycle progression in cancer cells. Sodium selenite causes cancer cells to

arrest at the S/G2 checkpoint *in vitro* and subsequently undergo apoptosis (Zeng and Combs, 2008).

Selenium is also required for optimal functioning of the immune system. Signs of selenium deficiency were first identified in patients receiving total parental nutrition (TPN). These patients experienced immunosuppression resulting from depressed antibody production. Supplementation with 200 μg of L-selenomethionine (SeMet) reversed this phenomenon, increasing antibody levels by a factor of 10 (Schrauzer and Surai, 2009). Many other pathological processes are associated with Se-deficiency: toxemia, nephropathy, edema, pre-eclampsia, retained placenta, hypertension, and stroke (Schrauzer and Surai, 2009).

Epidemiologic studies have demonstrated the protective effect of selenium against many diseases, including cancer. The observation was made as early as 1911, but disregarded due to the poorly understood toxicity profile of the element (Schrauzer and Surai, 2009). The most conclusive scientific evidence on the effects of Se-supplementation has been provided by two large, randomized, placebo-controlled trials in the United States.

The Nutritional Cancer Prevention Trial (NPC) was designed to analyze the effect of Se-supplementation of non-melanoma skin cancers (NMSC) (Clark et al., 1996). The study included 1312 patients with a history of NMSC recruited from the eastern seaboard, an area known to have Se-deficient soils. Patients were treated with 200 μg/d of selenium supplied by Se-enriched brewer's yeast or a placebo. Original analysis of the data (1983-1993) showed no effect of selenium treatment on NMSC. A significant difference was observed between the two treatment groups for several secondary endpoints. Se-supplementation significantly decreased both total cancer incidence and total cancer morality. The protective effect was site-specific for prostate, colorectal, and lung cancers, but a reduction of cancer risks was not seen at all sites (Clark et al., 1996). This study was later extended to cover data through 1996. A significant chemoprotective effect of Se-supplementation was still observed for total cancer and prostate

cancer incidence, but was not significant for lung or colorectal cancer. Additional analysis showed that the protective effects of Se-supplementation were greatest in males and in individuals who had low baseline plasma selenium concentrations (Duffield-Lillico et al., 2002).

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) was designed to assess the impact of selenium and vitamin E supplementation on prostate cancer risk (Klein et al., 2012). The study included 35,533 men of average prostate cancer risk divided into four treatment groups: Se-supplemented (200 μg/d L-selenomethionine), vitamin E-supplemented (400 IU/d), Se and vitamin E-supplemented, or placebo (Lippman, 2005). Se-supplementation did not have a significant impact upon prostate cancer risk. Vitamin E-supplementation increased the risk of prostate cancer by 17%, but selenium protected against this effect in the combined treatment group (Klein et al., 2012).

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is the most common myeloid leukemia. It affects 3.8 per 100,000 people, the prevalence rising with age (Estey, 2006). The median age of diagnosis is 70 years. AML is characterized by various chromosome abnormalities including aneuploidy, deletions, and balanced translocations of the long arm (q) of chromosomes (Estey and Dohner, 2006). AML is a common sequela to other cancers due to the cytogenetic damage during radiation and chemotherapy treatments. 10 to 15% of AML cases develop after cytotoxic chemotherapy treatment for solid cancers (Estey and Dohner, 2006).

AML is diagnosed when the bone marrow contains greater than 30% blasts of myeloid origin. Blasts are precursor cells that have lost the multipotent capability of stem cells. During normal hematopoiesis, multipotent hematopoietic stem cells (HSCs) give rise to all blood cell lineages (see Figure 6). HSCs are population of self-renewing cells in the bone marrow. HSCs daughter cells called multipotent progenitors (MPP) retain their multipotent capacity, but lose the

ability to self-renew (Giebel and Punzel, 2008). MPP give rise to the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP), which are the progenitors of the two major blood cell lineages. The myeloid lineage can be distinguished from the lymphoid lineage by the presence of the cell surface molecule CD34. CMPs differentiate into the granulocyte-macrophage progenitor (GMP) and the megakaryocyte-eythroid progenitor (MEP). GMPs give rise to monoblasts, the monocyte progenitor, and myeloblasts, and the common granulocyte progenitor. MEPs differentiate into megakaryocytes and the erythroid progenitor, burst forming units (BFU-E). BFU-E give rise to colony forming units (CFU-E) that give rise to large numbers of erythroblasts. After several stages of differentiation they ultimately form reticulocytes which mature into erythrocytes in the blood (Giebel and Punzel, 2008).

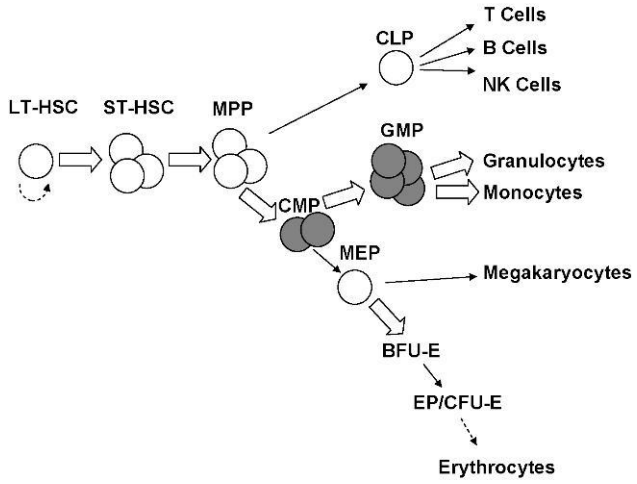

Figure 6. Normal hematopoiesis cascade.
Source: Chan G et al. 2009.

Leukemia stem cells (LSCs) display the same properties as other stem cells: self-renewing, multipotent, quiescent, and great proliferative potential. An accumulation of mutations in normal HSC or later progenitor cells that regain their self-renewal potential may produce LSCs ($CD34^{+}CD38^{-}CD123^{+}$) (Jordan, 2007). A two-hit model for the accumulation of mutations has been proposed. The first hit results in the constitutive activation of cell surface receptors, such as RAS and receptor tyrosine kinases. The constant proliferation signaling

results in the clonal expansion of the mutated progenitor cell (Moreau-Gachelin, 2006). The second hit inhibits myeloid differentiation. Failure to differentiate may be a result of gene fusions or over-expression of HOX genes. Proliferation of undifferentiated blasts retards normal hematopoiesis resulting in a deficiency of mature erythrocytes, monocytes, neutrophils, and platelets. Bone marrow failure and subsequent opportunistic infection is the primary cause of AML mortality.

Cytotoxic chemotherapy is the conventional treatment for AML. Patients are treated with a cocktail of an anthracycline drug and cytarabine (Estey and Dohner, 2006). Depending on cytogentic risk factors chemotherapy may be followed by stem cell transplant. Allogenic stem cell transplants reduce the risk of relapse by inducing graft-versus-leukemia effect, targeting of abnormal cancer cells by donor lymphocytes. Fatal complications arise in 10-25% of stem cell transplants. The goal of therapy is to achieve complete remission. Complete remission is defined as less than 5% blasts in the bone marrow and a recovery of hematopoiesis, indicated by a neutrophil count exceeding 1000 and a platelet count exceeding 100,000 (Estey and Dohner, 2006). Unfortunately, conventional therapies achieve lasting remission in less than 20% of patients (Rushworth et al., 2010).

Minimum residual disease maintained by LSCs leads to relapse. The quiescent state of LSCs, as opposed to the rapid proliferation of blast cells, contributes to their resistance to conventional chemotherapies. Additionally, LSCs exhibit insensitivity to apoptotic signals and the ability to efflux drugs (Jordan, 2007; Li-na et al., 2009). An important and unrealized therapeutic goal is the elimination of LSCs. Experimental AML therapies have been developed to directly target LSCs, but none have been approved for clinical use.

Immunotherapy targets molecules preferentially expressed on the surface of LSCs. In clinical trials anti-CD33 monoclonal (GO) antibody achieved a 30% remission rate (Li-na, 2009). However, GO also targets CD33 expressed on normal HSCs resulting in prolonged

cytopenia and immunosuppression following treatment. On the other hand, anti-CD47 antibody opsonizes LSCs enabling phagocytosis (Li-na et al., 2009).

Two important targets in LSCs are the constitutively active phosphatidylinositol 3 (PI3) kinase and NF-κB signaling pathways (Jordan, 2007). NF-κB is not activated in normal HSC. Two inhibitors of NF-κB have been shown to selectively destroy LSCs in culture. Parthenolide preferentially targets LSCs, but its usefulness as a therapeutic agent may be limited by its low water solubility. TDZD-8 (4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione) is a competitive inhibitor of glycogen synthase kinase (GSK)-3β. The mechanism of NF-κB inhibition is not understood, but it rapidly induces cell death in LSCs (Jordan, 2007).

15d-PGJ_2 may also be able to selectively destroy LSCs. Like the other prospective agents, 15d-PGJ_2 inhibits the NF-κB pathway. Our laboratory has shown that Δ^{12}-PGJ_2 and 15d-PGJ_2 both induce apoptosis in FV-LSCs (Hegde et al., 2011). 15d-PGJ_2 acts in a dose dependent manner, approaching maximum efficacy at concentrations of 25 nM. 15d-PGJ_2 has also proven effective *in-vivo*. FV-LSCs transplanted mice were rescued from splenomegaly following a week of daily treatment with 0.025 mg/kg 15d-PGJ_2 (Hedge et al., 2011).

Friend virus-induced erythroleukemia

In the laboratory, AML is modeled by murine erythroleukemia induced by the Friend virus complex. Friend virus (FV) is a complex of replicative-deficient spleen focus forming virus (SFFV) and replicative-competent Friend murine leukemia virus (F-MuLV). FV infection progresses through two stages (Subramanian et al., 2008). The first stage is characterized by acute erythrocytosis caused by proliferation of terminally differentiated erythrocytes in the spleen. The second stage is full-blown erythroleukemia characterized by rapid proliferation of undifferentiated erythroid cells.

Bone morphogenetic protein 4 (BMP4)-dependent stress erythropoiesis pathway is a

physiologic response that aides an animal to overcome tissue hypoxia resulting from severe anemia by rapidly producing new erythrocytes. Short-term reconstituting hematopoietic stem cells (ST-HSCs; $CD34^{+}Kit^{+}Sca^{+}Lin^{-}$) in the bone marrow give rise to dedicated stress BFU-E found in the spleen (Paulson et al., 2011). The replication of stress BFU-E is dependent on two signals: BMP4 upregulated in response to hypoxia-inducible factor-2α (HIF-2α) and the binding of stem cell factor (SCF) by the Kit receptor. Hedgehog signaling in the spleen microenvironment sensitizes BFU-E to BMP4 (Paulson et al., 2011).

The stress erythropoiesis pathway is activated during-FV infection when infected cells migrate from the bone marrow to the spleen and induce BMP4 expression. This produces a population of stress BFU-E in the spleen susceptible to FV infection. The *Fv2* gene in susceptible mice encodes a truncated form of the stem-cell tyrosine kinase receptor (sf-Stk). SFFV contains an *env* gene that encodes a 55 kDa glycoprotein (gp55). Constitutive activation of the sf-Stk and erythropoietin receptors (EpoR) through interaction with gp55 induces erythropoietin-independent proliferation and impairs differentiation of erythroid progenitors (Moreau-Gachelin, 2008).

Stress BFU-Es retain the ability to self-renew making them susceptible to transformation to LSCs. The proviral insertional activation of Spi1/Pu1 is essential for self-renewal of FV-LSCs. Spi/Pu1 works cooperatively with sf-Stk to block maturation of erythroid precursors producing a clonal leukemia (Hedge et al., 2012).

Statement of Problem

Based on the previous studies, we intend to show that Se-supplementation causes an upregulation of 15d-PGJ_2, which is responsible for the anti-carcinogenic effect of selenium in leukemia models. We cultured murine RAW 246.7 macrophages under Se-deficient and Se-supplemented conditions. Several inhibitors of the AA pathway, indomethacin, HQL-79, and CAY10526, were used to differentially block terminal prostaglandin synthesis. Indomethacin inhibits all cyclooxygenase activity inhibiting synthesis of all prostaglandins. HQL-79 and CAY10526 are selective inhibitors of H-PGDS and mPGES-1 blocking the production of PGD_2 and PGE_2, respectively. Lipid extracts were prepared from the supernatants of the treated RAW 246.7 cells. The extracts contained any lipid soluble molecules, such as prostaglandins, present in the supernatants.

The lipid extracts were used to treat LSCs *ex vitro*. Following a 48 hour incubation with the lipid treatments, the LSCs were assayed for viability. The studies were based on the hypothesis that apoptosis would greatly increase in LSCs treated with lipids from Se-supplemented cells in the absence of inhibitors and Se-supplemented cells in the presence of CAY10526. 15d-PGJ_2 was expected to be present in both of these samples because there was no inhibition of PGD_2 production and its downstream metabolites, the J series prostaglandins. As has previously been established by our laboratory, 15d-PGJ_2 induces apoptosis in LSCs. We hypothesized Se-deficient cells would produce insufficient 15d-PGJ_2 to induce apoptosis in LSCs. It was expected that indomethacin and HQL-79 would inhibit the terminal production of 15d-PGJ_2 thereby preventing apoptosis even in the presence of supranutritional levels of selenium.

Materials & Methods

Materials

The following chemicals were obtained from Sigma Aldrich Chemical Company (St. Louis, MO): hydrochloric acid, ethyl acetate (99.8% HPLC grade), anhydrous sodium sulfate, lipopolysaccharide (LPS; serotype 0111B4), and sodium selenite. The solvents toluene and hexane (95% HPLC grade) were obtained from EMD Chemicals (Billerica, MA). The following inhibitors were obtained from Cayman Chemical (Ann Arbor, MI): indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid; $C_{19}H_{16}ClNO_4$], HQL-79 [4-(diphenylmethoxy)-1–3-(1H-tetrazol-5-yl)propyl-piperidine; $C_{22}H_{27}N_5O$], and CAY10526 [4-(benzo[b]thiophen-2-yl)-3-bromo-5-hydroxy-dihydro-furan-2(3H)-one; $C_{12}H_7BrO_3S$]. Dulbecco's Modified Eagle's medium (DMEM) was obtained from Invitrogen (Carlsbad, CA). Hyclone (Logan, UT) supplied defined fetal bovine serum (FBS) with low levels of Se (6 nM).

Cayman Chemical supplied the antibodies prostaglandin D synthase (hematopoietic-type; mouse) polyclonal antiserum and prostaglandin E synthase (microsomal) polyclonal antibody. Anti-glutathione peroxidase 1 polyclonal antibody was purchased from Abcam (Cambridge, MA). Glyceraldehyde 3-phosphate dehydrogenase mouse monoclonal antibody was supplied by Research Diagnostics Inc. (Flanders, NJ).

Cell Culture and Treatments

RAW 264.7 cells, a mouse leukemic monocyte macrophage cell line, were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM), supplemented with FBS (5%), penicillin-streptomycin solution (1%), and L-glutamine solution (1%) and maintained in a 37°C incubator in a humidified 5% carbon dioxide atmosphere. Cell cultures were maintained in T-75 flasks passaging cells once 80% confluence was reached.

For experimentation, cells were seeded onto 10 cm^2 plates at a concentration of $2.5x10^6$ cells per plate in 5 mL of 5% FBS DMEM. Plated cells were treated immediately with either 5 μL DMSO, as a vehicle control, 10μM indomethacin, 25 μM HQL-79, or 20 μM CAY10526 and incubated at 37°C for 1 hour. After 1 hour cells, 100 ng/ml LPS was added to complete media and incubated for 2 hours after which cells were washed with DMEM. Treatments were again added to the cells in 5 mL of fresh media.

Following a 30 minute incubation at room temperature Se treatments were added. For each treatment group, a plate of Se-deficient and Se-supplemented cells was used. Defined FBS containing 6 pmol/mL of Se, was the only source of Se for Se-deficient cells (Vunta, 2007). Se-supplemented cells were treated with selenium selenite (100 nM or 250 nM).

Cell supernatants were collected individually daily for three days, for a total volume of 15 mL each, and stored at -80°C. After collection of supernatants, treatments were again added to the cells in 5 mL of fresh media. After the final supernatant collection, each plate was washed phosphate buffered saline (PBS). The plates were then scraped and the cells collected in 1 mL of PBS. The tubes were centrifuged (7500 rpm, 4°C, 5 min) and the supernatant discarded to obtain cell pellets, which were stored at -80°C until future use.

Lipid Extraction

Lipid extracts were prepared from the collected cell supernatants. Supernatants were acidified with 1 to 2 drops of 12N HCl (until color change). To the 15 mL of acidified supernatant, 15 mL of ethyl acetate: hexane (1:1) mixture was added in separatory funnel. The solution was mixed vigorously and the phases allowed to separate. The lower aqueous phase was removed and discarded. The upper organic phase was filtered through anhydrous sodium sulfate pre-saturated with ethyl acetate and collected in 20 mL brown glass vials. Hexane:ethylacetate was evaporated with argon gas and immediately reconstituted in 5 mL ethyl

acetate and stored at -20°C until further use.

Cell Viability Assay

Spleen cells from Friend Virus infected mice were sorted for $M34^{+}Sca1^{+}Kit^{+}$ leukemic stem cells (LSCs) by flow-cytometry. Sorted cells were plated in 2 mL of methylcellulose stem cell media supplemented with 200 ng/ml sonic hedgehog (sHH), 50 ng/ml SCF, and 15 ng/ml BMP4 per well in a 36 well plate. To each well was added 200 μL of RAW 246.7 cell supernatant lipid extracts reconstituted in PBS. Lipid extract treatments were prepared from 200 μL of sample stored in ethyl acetate. The samples were blown down under argon gas, and immediately reconstituted in 200 μL of warm PBS. Treated cells were incubated for 36 hours. Cell viability was measured by flow cytometry for GFP-FITC.

Electrophoresis and Immunoblotting

Proteins for western blot analysis were prepared by resuspending cell pellets in 100 μL of mammalian protein extract reagent (MPER; Thermo Pierce) containing the protease inhibitors aprotinin, pepstatin, and phenylmethanesulfonylfluoride (PMSF), pipette to mix thoroughly, and incubated on ice for 5 minutes. Samples were then centrifuged (14000g, 4°C, 10 min) and the supernatants containing cytoplasmic proteins transferred to new tubes. The cell pellet was discarded.

Protein estimation was performed using the Thermo Scientific Pierce 660 nm Protein Assay reagent and pre-diluted bovine serum albumin (BSA) standards (25-2000 μg/mL). 3 μL of each protein sample was diluted in 7 μL of Milli-Q water for a total volume of 10 μL per well, and incubated at room temperature for 5 minutes. Absorbance was measured at 620 nm using a Spectra Count plate reader (Packard-Perkin Elmer Instruments). A standard curve constructed from the standard values was used to estimate protein concentration (μg/mL).

Based on protein estimates samples were prepared for a protein loading volume of 10 μg

or 25 µg. All samples contained 6X SDS-PAGE loading dye and Milli-Q water such that the final sample volumes were equal. Samples were heated on a VWR heat block at 100°C for 5 minutes and stored at -80°C for future use.

Samples were electrophoresed on a 0.75mm 12% SDS-polyacrylamide gel in 5% SDS-PAGE running buffer, containing 15.1 Tris base, 72 g glycine, and 5 g SDS per 1000 mL of Milli-Q water for 60 minutes at 120 V. The gel was then transblotted to a nitrocellulose membrane using a 1X transfer buffer, containing 12.1 Tris base, 57 g glycine, and 800 mL of methanol in 4 L of water at 110 V for 90 minutes.

The membrane was blocked using a 5% solution of non-fat powdered milk in 1X Tris-buffered saline containing 0.05% Tween-20 (0.1% TBST) for 1 hour. Primary antibody was then added to the milk solution and incubated overnight at 10°C (see Table 1), with the exception of GAPDH which was only incubated for 1 hour at room temperature. Following probing the membrane was washed three times with 0.1% TBST buffer for 10 minutes per wash. The membrane was then probed with a complementary secondary antibody at room temperature for 1 hour, after which the membrane was again given three washes. After development the membrane was treated with 10 mL of Restore Membrane stripping buffer (purchased from Thermo Pierce) and incubated at 37°C at 100rpm for 1 hour, given three 10 minute washes with 0.1% TBST, and then re-blocked and probed with a different antibody.

Table 1. Antibody concentrations for western blot technique

Antibody	Host	Protein Conc (µg)	1° Ab conc	2° Ab conc	MW (kDa)
GPX	Rabbit	10	1:2500	1:2500	22
H-PGDS	Rabbit	10	1:1000	1:2500	23
GPX	Rabbit	25	1:2000	1:5000	22
H-PGDS	Rabbit	25	1:2000	1:2500	23
mPGES-1	Rabbit	25	1:1000	1:5000	16
GAPDH	Mouse	varied	1:600,000	1:5000	36

Autoradiography and Densitometry

In order to visualize the antibodies bound to the proteins on the membrane, a chemiluminescence substrate assay kit from Pierce chemical was used. Chemiluminenscence was visualized with HyBlot CL autoradiography film developed in an automated film processor. Protein bands were analyzed using the Image J densitometric analysis program (National Institutes of Health, MD). To control for differences in protein loading concentrations, protein values were normalized against the ubiquitously expressed protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Thin Layer Liquid Chromatography

Thin layer liquid chromatography (TLC) was performed on lipid extracts. To prepare samples 0.5 mL of lipid extracts from Se-deficient and Se-supplemented RAW 246.7 cells untreated or treated with indomethacin or HQL-79 in ethyl acetate were evaporated under Argon gas and reconstituted in 10 μL of ethyl acetate. Samples were loaded on a Whatmen 250 μm silica gel plate on aluminum support (Whatman, UK) with 1μg of 15d-PGJ_2 and 1μg Δ12-PGJ_2 as standards. The plate was allowed to run 6.0 cm in a toluene : acetone : acetic acid (80:20:2) motile phase. The plate was then developed in an iodine chamber for 90 minutes for visualization of lipid spots.

15d-PGJ_2 Quantification by Mass Spectometry

Lipid extracts were analyzed for the presence of 15d-PGJ_2 by direct infusion into a triple quadruple mass spectrometer (API 2000, ABI SCIEX) in the negative electrospray ionization mode. Samples were analyzed in a 70:30 ethanol : H_2O solvent system with 0.1% acetic acid. 15d-PGJ_2 at concentrations of 25 ng, 5 ng, and 1 ng was used as a standard. 50 μL samples of lipid extracts from Se-deficient and Se-supplemented (250 nM) RAW 246.7 cells were analyzed. Samples were run for 35 minutes; 15d-PGJ_2 elutes at 41.467 minutes. The peak area

corresponding to 315 MW was used to quantify 15d-PGJ_2 concentration.

Results

Glutathione Peroxidase Expression is an Indicator of Cellular Selenium Status

RAW 246.7 cells were cultured in Se-deficient or Se-supplemented (100 nM sodium selenite) media. Prior to treatment cells were stimulated with LPS for two hours to induce oxidative stress. Glutathione peroxidase (GPX) is an important enzyme in protecting cells from oxidative damage. GPX is a selenoprotein containing the amino acid selenocysteine. As demonstrated in previous studies by our laboratory GPX-1 is a highly sensitive dose-dependent indicator of cellular selenium status in RAW 246.7 macrophages (Zamamiri-Davis et al., 2002). Immunoblot analysis of cell lysates with anti-GPX antibody was preformed to confirm selenium status (see Figure 7). No GPX expression is observed in selenium deficient cells. The GPX band is clearly visible in Se-supplemented cells.

RAW 246.7 cells were treated with indomethacin, HQL-79, and CAY10526, inhibitors of the arachidonic acid metabolism enzymes cyclooxygenase, H-PGDS, and mPGES-1, respectively. The presence of inhibitors did not affect cellular selenium status. A replicate of this experiment with RAW 246.7 cells showed similar results (see Figure 8). Across all treatment groups, GPX expression was observed only in the presence of Se-supplementation.

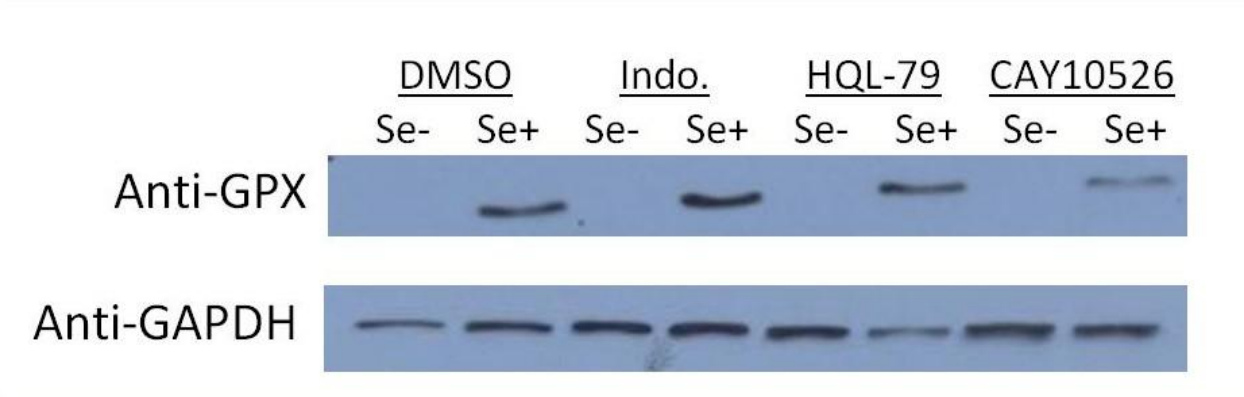

Figure 7. Effect of Se-supplementation on GPX expression – Trial 1. LPS-stimulated RAW 246.7 macrophages were cultured for 3 days under Se-deficient or Se-supplemented (100 nM) conditions in the presence of indomethacin (10 μM), HQL-79 (25 μM), or CAY10526 (20 μM). Cytoplasmic lysates were used for western blot analysis. GPX expression was only observed in Se-supplemented cells. GAPDH expression is shown as a loading control.

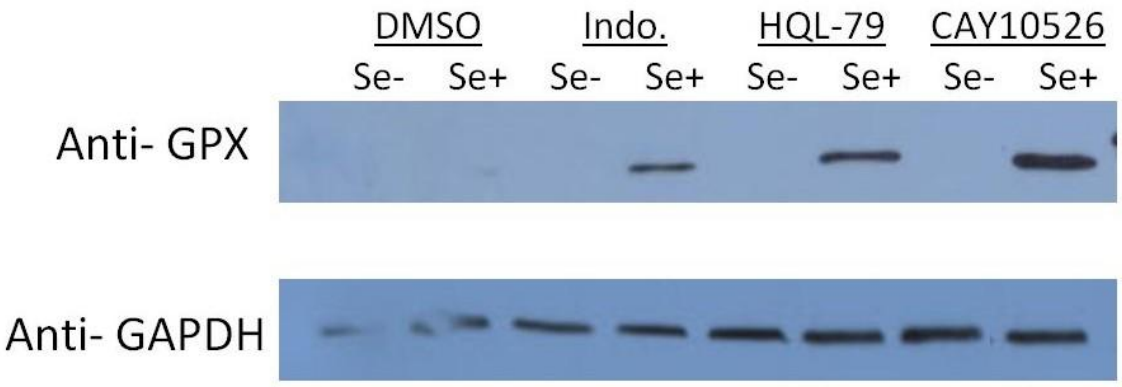

Figure 8. Effect of Se-supplementation of GPX expression – Trial 2. LPS-stimulated RAW 246.7 macrophages were cultured for 3 days under Se-deficient or Se-supplemented (250 nM) conditions in the presence of various inhibitors. Cytoplasmic lysates were used for western blot analysis. GPX expression was only observed in Se-supplemented cells. GAPDH expression is shown as a loading control.

Hematopoietic Prostaglandin D_2 Synthase Expression is Unregulated in Response to Selenium Supplementation

RAW 246.7 cells were treated with various inhibitors of the arachidonic acid (AA) metabolic pathway. Indomethacin is an inhibitor of both cyclooxygenase 1 and 2 (COX 1 and 2). HQL-79 is a selective inhibitor of hematopoietic prostaglandin D synthase (H-PGDS) action. CAY10526 is a selective inhibitor of microsomal prostaglandin E synthase (mPGES-1) expression.

H-PGDS converts PGH_2 to PGD_2, which is the precursor of the J series of prostaglandins. To visualize H-PGDS expression an immunoblot of RAW 246.7 cell lysates was probed with anti-H-PGDS antibody (see figure 9). H-PGDS expression is highest in HQL-79 and CAY10526 treated cells. Both inhibitors alter the conversion rates of PGH_2 to PGD_2 and its downstream metabolites. HQL-79 does not inhibit H-PGDS expression, but inhibits its enzymatic activity. CAY10526 is a selective modulator of mPGES-1 expression. Indomethacin, which non-selectively inhibits the entire prostaglandin synthesis cascade, induces greater expression of H-PGDS than in untreated cells. Within each treatment group H-PGDS is more highly expressed in

the Se-supplemented cells than the Se-deficient cells (see figure 10). Se-supplementation results in a statistically significant increase in H-PGDS expression (P= 0.012).

Figure 9. The effect of Se-supplementation on H-PGDS expression – Trial 1. LPS-stimulated RAW 246.7 macrophages were cultured for 3 days under Se-deficient or Se-supplemented (100 nM) conditions in the presence of indomethacin (10 μM), HQL-79 (25 μM), or CAY10526 (20 μM). Cytoplasmic lysates were used for western blot analysis. GAPDH expression is displayed as a loading control.

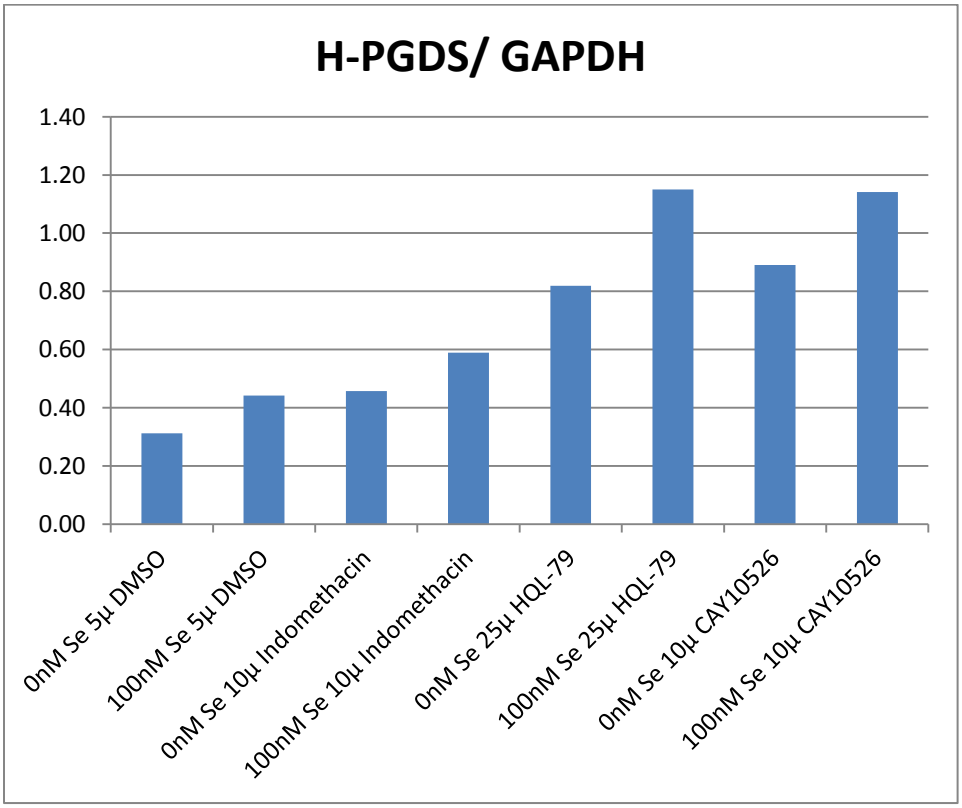

Figure 10. Densitometry results for H-PGDS western blot – Trial 1. Expression of H-PGDS in LPS, selenium, and inhibitor treated RAW 246.7 cells normalized to GAPDH expression.

The same H-PGDS expression pattern was observed when the experiment was replicated (see Figure 11). Highest H-PGDS expression was observed in HQL-79 and CAY10526 treated samples. Lowest expression of H-PGDS was observed in untreated controls; intermediate expression of H-PGDS was observed in indomethacin treated samples. Again within each treatment group Se-supplemented cells exhibited the highest H-PGDS expression (P=0.097) (see Figure 12).

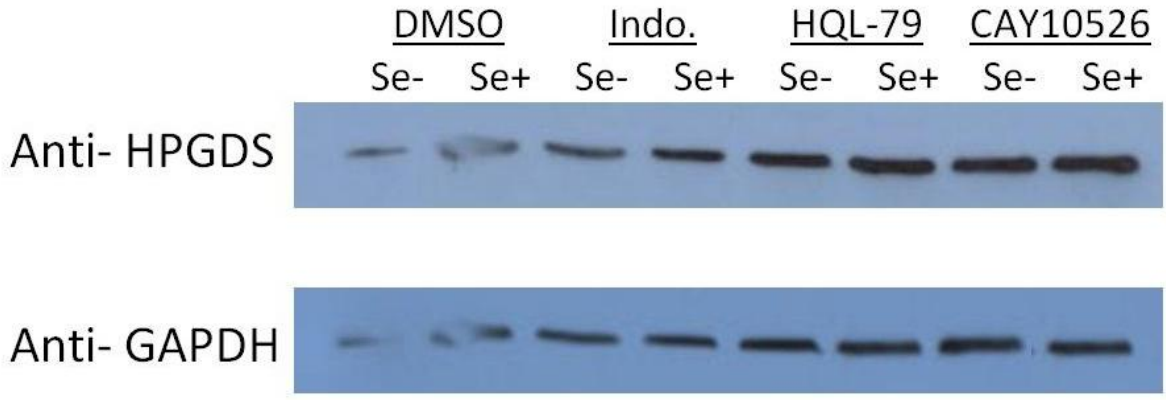

Figure 11. The effect of Se-supplementation on H-PGDS expression – Trial 2. LPS-stimulated RAW 246.7 macrophages were cultured for 3 days under Se-deficient or Se-supplemented (250 nM) conditions in the presence of various inhibitors. Cytoplasmic lysates were used for western blot analysis. GAPDH expression is displayed as a loading control.

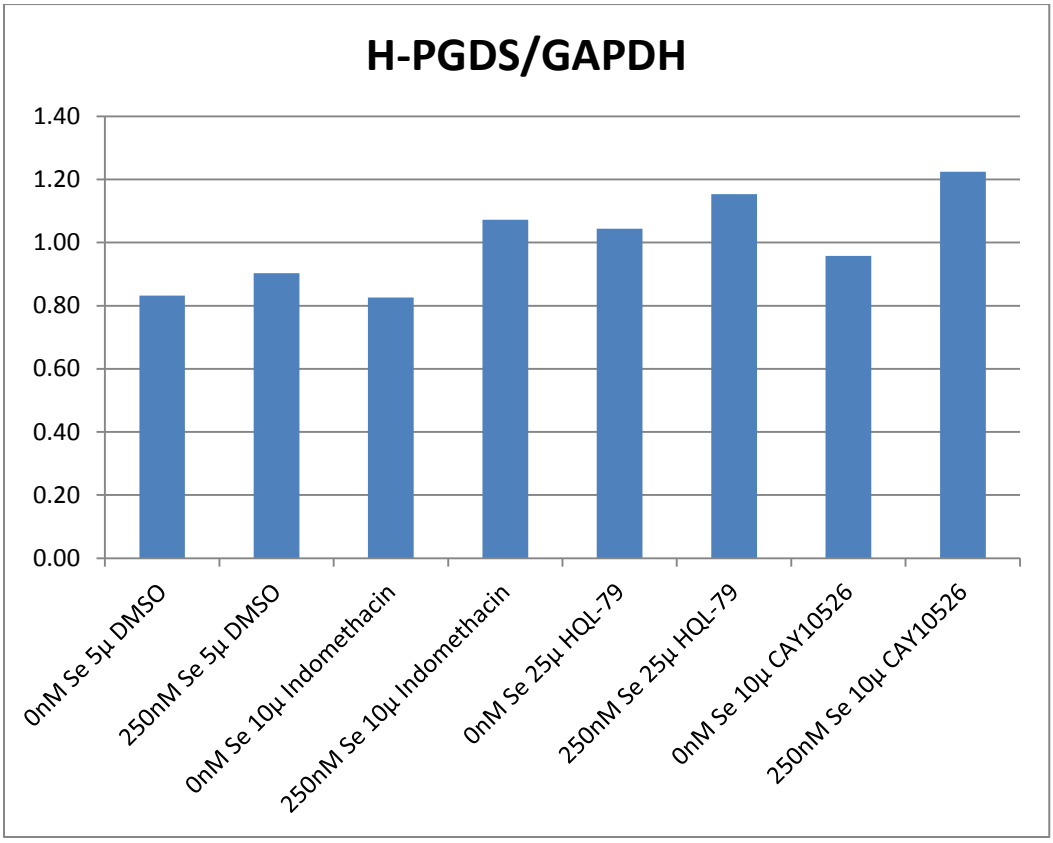

Figure 12. Densitometry results for H-PGDS western blot – Trial 2. Expression of H-PGDS in RAW 246.7 cells in the presence of selenium and inhibitors normalized to GAPDH expression.

A metabolite of PGD_2 induces apoptosis in leukemic stem cells

Supernatants were collected from RAW 246.7 cells grown under Se-deficient or Se-supplemented conditions in the presence of indomethacin, HQL-79, or CAY10526. Lipid extracts were purified from the supernatants. LSCs were treated with 200 µL of lipid extracts and incubated for 36 hours. LSCs were then assessed for viability by flow cytometry. All presented viability data is the average of two trials.

Treatment of LSCs with PBS as negative control established a baseline viability of 93.1%. Treatment with lipid extract from Se-deficient RAW 246.7 cells did not alter LSC viability from baseline (92.1%). Se-supplemented (100 nM) lipid treatment caused a significant increase in apoptosis; only 4.25% of LSCs survived. Se-supplementation increased the synthesis of a pro-apoptotic compound in RAW 246.7 macrophages. To investigate the source of this pro-

apoptotic compound RAW 246.7 cells were treated with inhibitors of the arachidonic metabolism pathway. Indomethacin, an indiscriminate inhibitor of cyclooxygenase, inhibited apoptosis by both Se-deficient (86.95%) and Se-supplemented (92.55%) lipid extracts. Prostaglandins are necessary for the induction of apoptosis in LSCs. Specific inhibitors of prostaglandin synthesis were used to investigate which prostaglandins were critical for apoptotic activity. CAY10526, an inhibitor of mPGES-1, inhibits the synthesis of PGE_2. CAY10526 did not block apoptosis (10.05%) in LSCs treated with lipid extract from Se-supplemented cells. Elevated apoptosis (87.85%) was not observed with lipids from CAY10526 treated Se-deficient cells. PGE_2 is not responsible for the induction of apoptosis in LSCs. HQL-79 is an inhibitor of H-PGDS, blocking the synthesis of PGD_2 and its downstream metabolites the J-series prostaglandins. Lipid extracts from both Se-deficient and Se-supplemented cells treated with HQL-79 failed to induce apoptosis in LSCs (86.7% and 90.8% viability, respectively). A metabolite of PGD_2 is responsible for the pro-apoptotic activity induced by Se-supplemented cells (see Figure 13).

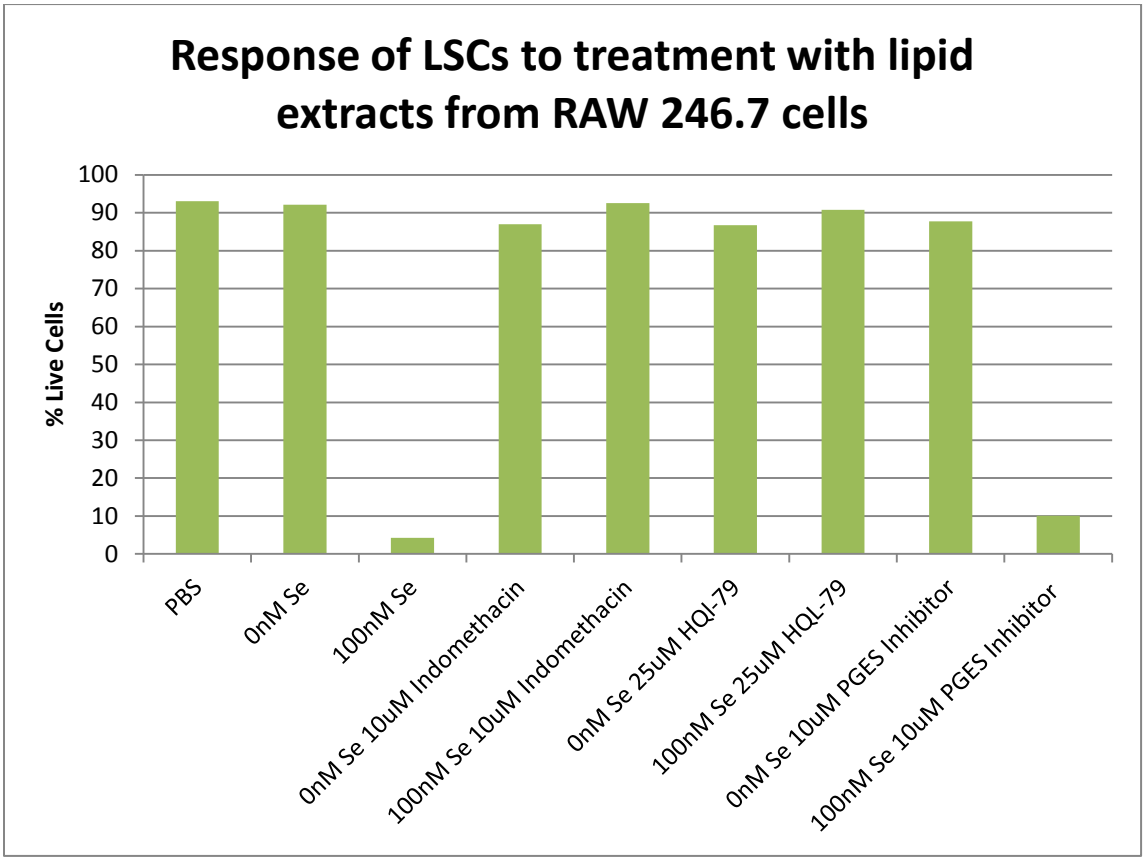

Figure 13. Survival of LSCs following treatment with lipid extracts from RAW 246.7 cells – Trial 1. Supernatants from LPS-stimulated RAW 246.7 macrophages cultured under Se-deficient or Se-supplemented (100 nM) conditions in the presence of indomethacin (10 μM), HQL-79 (25 μM), or CAY10526 (20 μM) were collected over 3 days. LSCs were treated with lipid extracts (200 μL) prepared from the RAW 246.7 cell supernatants and incubated for 36 hours. Cell survival was determined by fluorescent sorting on a flow cytometer. The displayed values are an average of two trials.

As proof of principle we replicated the apoptotic assay, treating LSCs with lipid extracts derived from the supernatants of Se-deficient and Se-supplemented (250 nM) RAW 246.7 cells treated with various inhibitors (see figure 14). Once again the lipid extract from untreated Se-supplemented RAW 246.7 cells showed a great increase in the number of apoptotic cells. Only 1.6% of LSCs treated with lipids from Se-supplemented cells were viable after 48 hours. No change in the rate of apoptosis was observed as a result of any of the other lipid treatments. LSC viability following lipid treatment from Se-deficient cells treated with DMSO, indomethacin, HQL-79, and CAY10526 was 84.65%, 85.35%, 85.05%, and 84.9% respectively. Following

lipid treatment from Se-supplemented cells treated with indomethacin, HQL-79, and CAY10526, LSC viability was 85.25%, 84.45%, and 85.4% respectively.

Based on the action of the inhibitor and prior experimentation, the lipid extract from Se-supplemented CAY10526 treated RAW 246.7 cells was expected to increase the number of apoptotic cells. Western blot for mPGES-1 shows that the CAY10526 used to treat the cells had lost its efficacy (see figure 15). mPGES-1 should not be expressed in the presence of CAY10526.

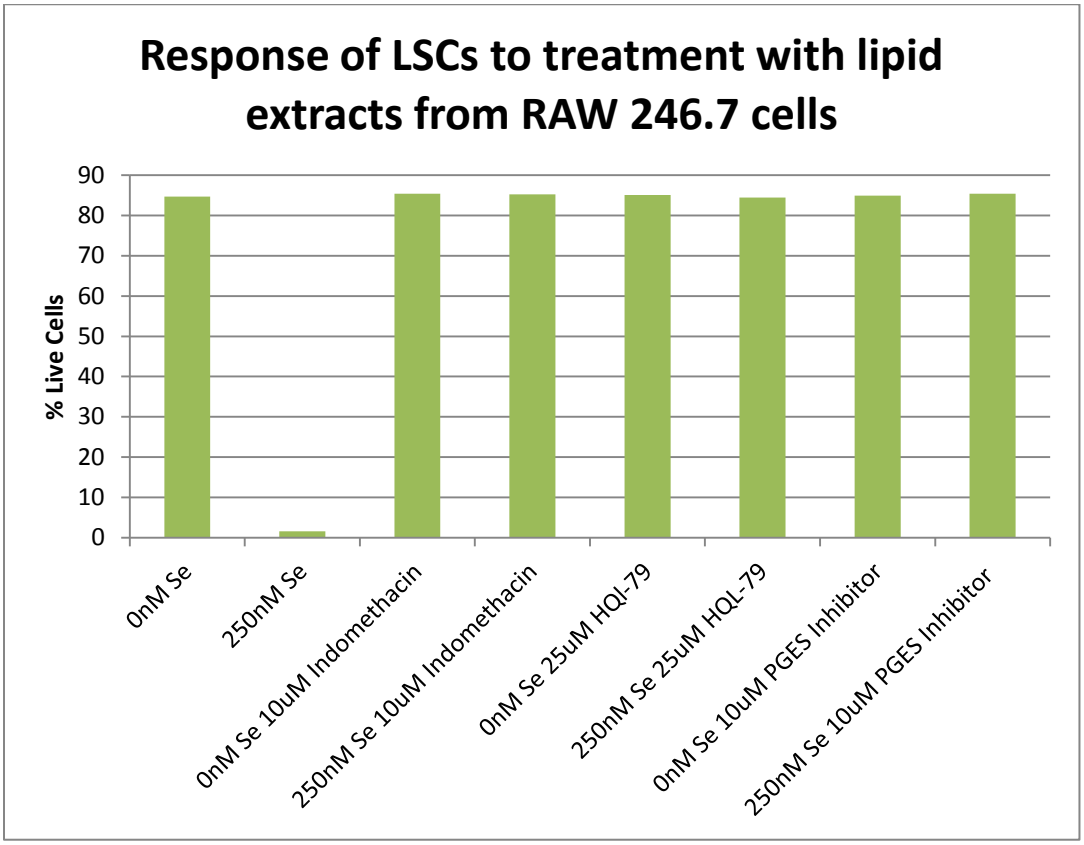

Figure 14. Survival of LSCs following treatment with lipid extracts from RAW 246.7 cells – Trial 2. Supernatants from LPS-stimulated RAW 246.7 macrophages cultured under Se-deficient or Se-supplemented (250 nM) conditions in the presence of indomethacin (10 μM), HQL-79 (25 μM), or CAY10526 (20 μM) were collected over 3 days. LSCs were treated with lipid extracts (200 μL) prepared from the RAW 246.7 cell supernatants and incubated for 36 hours. Cell survival was determined by flow cytometry. The displayed values are an average of two trials.

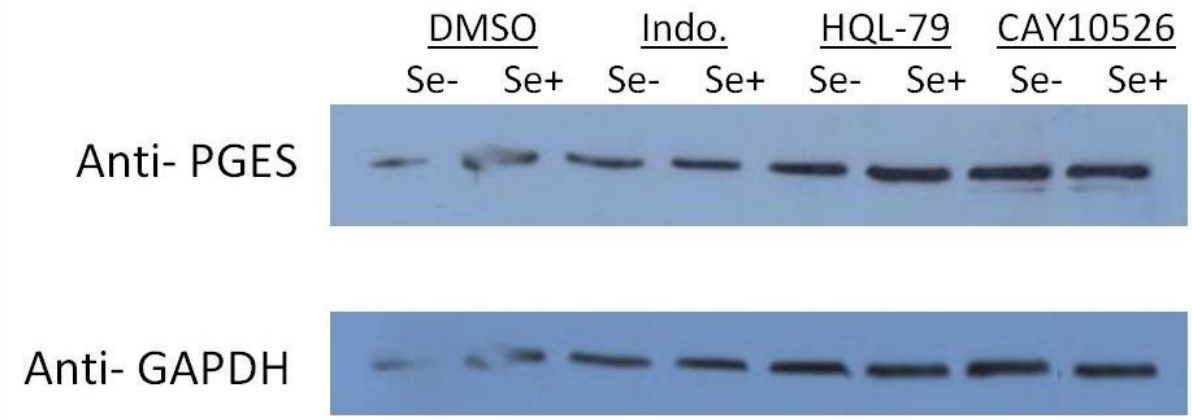

Figure 15. The expression of mPGES-1 in the presence of CAY10526 – Trial 2. LPS-stimulated RAW 246.7 macrophages were cultured for 3 days under Se-deficient or Se-supplemented (250 nM) conditions in the presence of indomethacin (10 μM), HQL-79 (25 μM), or CAY10526 (20 μM). Cytoplasmic lysates were used for western blot analysis. GAPDH expression is shown as a loading control.

Detection of PGJ_2 metabolites by thin layer liquid chromatography

500 μL of lipid extracts, from Se-deficient and Se-supplemented RAW 246.7 cells treated with indomethacin or HQL-79, concentrated to 10 μL in ethyl acetate were loaded on a 250 μm silica gel plate. Thin layer liquid chromatography (TLC) was performed using a toluene : acetone : acetic acid (80:20:2) motile phase. Good separation of the standards 15d-PGJ_2 (R_f= 0.51) and Δ12-PGJ_2 (R_f= 0.31) was achieved (see Figure 16). Standards at a volume of 1 μg were clearly visible with iodine staining. Standards at a volume of 0.5 μg were detectable (data not shown). No bands corresponding to the standards, 15d-PGJ_2 or Δ12-PGJ_2, were observed in the lipid extract samples. Any PGJ_2 metabolites present must be at concentrations less than 2.0 μM. Two spots corresponding to an unidentified lipid (R_f= 0.45) were observed in the lipid extracts from indomethacin treated RAW 246.7 cells, which need to be further characterized.

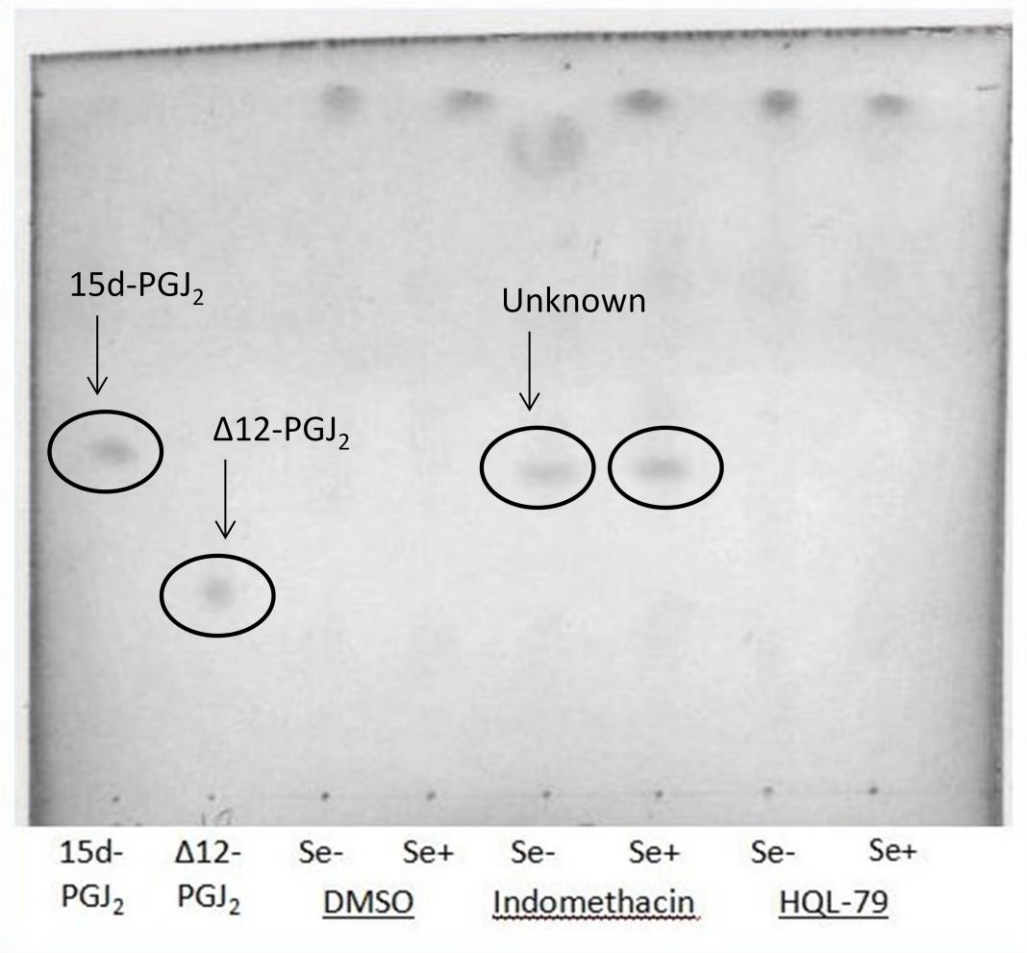

Figure 16. Thin layer liquid chromatography of lipid extracts from RAW 246.7 macrophages. Lipid extracts were prepared from the supernatants of LPS-stimulated RAW 246.7 macrophages cultured under Se-deficient or Se-supplemented (250 nM) conditions in the presence of indomethacin (10 μM) or HQL-79 (25 μM) for 3 days. 15d-PGJ_2 (1 μg) and Δ12-PGJ_2 (1 μg) standards were loaded on a 250 μm silica gel TLC plate. 500 μL of each lipid extract concentrated to 10 μL in ethyl acetate was loaded on the plate and run using a toluene:acetone:acetic acid (80:20:2) motile phase. The plate was developed in an iodine chamber for 90 minutes.

Quantification of 15d-PGJ_2 by mass spectrometry

Lipid extracts from Se-deficient and Se-supplemented (250 nM) RAW 246.7 cells were analyzed for the presence of 15d-PGJ_2 by MS/MS. 15d-PGJ_2 eluted at 31.467 minutes. Purified 15d-PGJ_2 was used as a standard; the largest peak corresponded to 315 MW. A larger peak was observed in the Se-supplemented sample than the Se-deficient sample (see Table 2). The peak area for the Se-supplemented sample was slightly less than the area observed for 5 ng 15d-PGJ_2. Peaks for 1 ng of 15d-PGJ_2 were not detectable above background. This technique is unable to resolve the identity of different compounds eluting at the same time point.

Table 2. **Mass spectrometry data for lipid extracts from Se-deficient and Se-supplemented RAW 246.7 cells**. Lipid extracts were prepared from the supernatants of LPS-stimulated RAW 246.7 macrophages cultured under Se-deficient (O nM) or Se-supplemented (250 nM) conditions for 3 days. Lipid extracts were analyzed by MS/MS for the presence of 15d-PGJ_2 measured at 31.467 minutes and 315 MW. 15d-PGJ_2 (25 and 5 ng) was used as a standard.

Sample	Peak Area (10^5)
25 ng 15d-PGJ_2	10.2330
5 ng 15d-PGJ_2	3.8113
0 nM Se	2.3048
250 nM Se	3.0610

Discussion

Conventional treatments of AML achieve lasting remission in less than 20% of patients (Rushworth, 2010). Standard treatment is parenteral administration of chemotherapeutic agents. These agents indiscriminately kill rapidly dividing cells, such as malignant blast cells as well as mitotically active healthy cells, accounting for many of the negative side effects of chemotherapy. LSCs remain quiescent in the bone marrow and thus escape targeting. Self-renewing LSCs maintain a disease reservoir within the bone marrow; at any point these progenitor cells may become active leading to blast crisis. In order to achieve complete remission of AML LSCs must be destroyed.

Currently there are no approved therapies for targeting LSCs, but several experimental molecules have been proposed. Immunotherapy has been used to target CD33 and CD47, but is not selective for LSCs (Jordan, 2007). Inhibitors of the constitutively active NF-κB signaling pathway can selectively induce apoptosis in LSCs, because NF-κB is not expressed in normal HSCs. NF-κB inhibitors include the synthetic drugs, parthenolide and TDZD-8, and the endogenously produced 15d-PGJ_2 (Jordan, 2007; Hedge et al., 2011). 15d-PGJ_2 treatment selectively induced apoptosis in LSCs ex vivo and prevented splenomegaly and the development of disease in FV-LSC transfected mice (Hedge et al., 2011). It appears that inhibitors of NF-κB alone are not enough to induce apoptosis; pro-apoptotic molecules must concomitantly induce oxidative stress in LSCs (Hassane et al., 2008).

In order to test the ability of Se-supplementation to induce apoptosis in LSCs we created lipid treatments from cultured RAW 246.7 macrophage supernatants. Lipid extracts from the supernatants of cultured cells contain lipid-derived signaling molecules, including prostaglandins, secreted by the cells. Lipid extracts from Se-deficient cells did not alter the rate of apoptosis in LSCs as compared to the negative control. Conversely, lipid extracts from Se-supplemented cells greatly increased the rate of apoptosis in LSCs. Since the lipid extracts from

Se-deficient cells did not affect LSCs, a lipid soluble mediator only expressed in Se-supplemented cells must be responsible for inducing apoptosis in LSCs.

The arachidonic acid pathway is a complex metabolic pathway with many regulatory points. Specific inhibitors of inducible prostaglandin synthases can be used to alter the terminal prostaglandin profile. Indomethacin, HQL-79, and CAY10526 inhibit the catalytic activity of COX, H-PGDS, and mPGES-1, respectively. Indomethacin blocks the synthesis of all prostaglandins and inhibits the pro-apoptotic effect of Se-supplementation. More selective inhibitors were used to determine which prostaglandin is essential for apoptotic activity in LSCs. Inhibition of mPGES-1 had no effect on apoptotic activity; lipids from Se-supplemented cells induced apoptosis while extracts from Se-deficient cells failed to induce apoptosis. This experiment showed that PGE_2 is not required for apoptotic activity. Inhibition of H-PGDS blocked the apoptotic activity of lipid extracts from Se-supplemented macrophages. Because apoptotic activity was blocked by the inhibition of H-PGDS, PGD_2 or one of its downstream metabolites, PGJ_2, Δ12-PGJ_2, or 15d-PGJ_2 must be responsible for inducing apoptosis. 15d-PGJ_2 has been found previously to induce apoptosis in LSCs (Hedge et al., 2011). Mass spectrometry results confirmed that Se-supplemented lipid extract contained a higher concentration of 15d-PGJ_2 than Se-deficient extract.

15d-PGJ_2 has been shown to induce apoptosis in LSCs through both PPARγ-dependent and PPARγ-independent pathways in different cell lines. It is a cyclopentenone prostaglandin with a highly reactive α, β-unsaturated ketone that can undergo Michael addition reactions with the cysteine thiol groups of cytosolic proteins (Surh et al., 2011). 15d-PGJ_2 is a potent endogenous ligand for PPARγ through which it acts as a negative regulator of AP-1, STAT, and NF-κB (Ricote et al., 1998). 15d-PGJ_2 can also inhibit the transcriptional activity of NF-κB in a PPARγ-independent manner through direct inhibition of IKK (Rossi et al., 2000). Another PPARγ-independent mechanism involves the 15d-PGJ_2 stimulated produced of ROS and the

downstream activation of executioner caspases and apoptosis.

15d-PGJ_2 induced apoptosis in LSCs does not appear to work through a PPARγ, NF-κB, or ROS mediated pathway. A synthetic PPARγ agonist, rosiglitazone, failed to induce apoptosis in LSCs (Hedge et al., 2011). Δ12-PGJ_3 derived from eicosapentenoic acid (EPA) is similar in structure and activity to Δ12-PGJ_2 and 15d-PGJ_2 with the addition of a *cis* double bond. Physiologic levels of Δ12-PGJ_3 fail to elicit PPARγ activity. Additionally, Δ12-PGJ_3 did not affect the NF-κB pathway. Δ12-PGJ_3 induces apoptosis in LSCs in a PPARγ and NF-κB-independent manner. It can be assumed that 15d-PGJ_2, which mimics the apoptotic activity of Δ12-PGJ_3, likewise acts independent of PPARγ and NF-κB pathways. Concentrations of 4 to 20 μM of 15d-PGJ_2 are necessary to stimulate ROS production (Shin et al., 2009; Han et al., 2007; Chen et al., 2005). Less than 5 ng of 15d-PGJ_2, as quantified by MS/MS, is sufficient to induce apoptosis in LSCs. Nanomolar concentration of Δ12-PGJ_3 did not induce ROS production (Hedge et al., 2011). In the absence of ROS production nucleophilic addition of reactive 15d-PGJ_2 to redox-sensitive thiol groups of transcription factors can signal oxidative stress (Surh et al., 2011).

15d-PGD_2 selectively up-regulates the transcription of p53 mRNA in LSCs (Hedge et al., 2011). In cells lacking functional p53 Δ12-PGJ_3 failed to induce apoptosis. p53 activity is essential for the apoptotic activity observed in LSCs. 15d-PGJ_2 is known to act through direct modification of ATM to upregulate p53 expression (Kobayashi et al., 2006). An ATM inhibitor blocked the increase in p53 and apoptosis caused by Δ12-PGJ_3 (Hedge et al., 2011). ATM-dependent up-regulation of p53 is the most likely mechanism for 15d-PGJ_2-induced apoptosis in LSCs.

Stat3 is a critical transcription factor in HSC. Phosphorylation of a critical tyrosine residue leads to transcription of genes that play a role cell cycle regulation, angiogenesis, and cell survival. Upstream oncoproteins constitutively activate Stat3 in many AML cell lines and

increases resistance to chemotherapy, associated with poor prognosis. A synthetic inhibitor of Stat3 activation, C188-9, induces apoptosis in AML cell lines (Redell et al., 2011). 15d-PGJ_2 inhibits the phosphorylation of Stat3 by leukemia induction factor (LIF) in embryonic stem cells (Rajasingh and Bright, 2006). A similar mechanism may occur in LSCs.

LSCs do not produce 15d-PGJ_2. The production of J series prostaglandins, including 15d-PGJ_2, is dependent upon the availability of the precursor molecule PGD_2. PGDS catalyzed conversion of PGH_2 to PGD_2 is the rate-limiting step in the synthesis of 15d-PGJ_2. H-PGDS is highly expressed in macrophages (Herlong and Scott, 2006).

Supplementation with 100 or 250 nM selenium upregulated the expression of H-PGDS in RAW 246.7 cells in comparison to Se-deficient cells. These results are supported by previously published work by our lab (Gandhi et al., 2011). Se-supplementation of RAW 246.7 cells upregulates H-PGDS expression in a dose-dependent fashion until reaching saturation between 150 and 250 nM selenium. Concentrations of 15d-PGJ_2 rose correspondingly with H-PGDS levels. Transfection of RAW 246.7 cells with a mutated selenophosphate synthetase 2 (SPS2) gene showed that incorporation of selenium into selenoproteins is critical for the expression of H-PGDS (Gandhi et al., 2011). No decrease in mPGES-1 expression in response to Se-supplementation was observed. It has been previously reported that Se-supplementation causes a dose-dependent downregulation of mPGES-1 expression (Gandhi et al., 2011).

The selenium induced upregulation of H-PGDS expression is PPARγ-dependent (Gandhi et al., 2011). The promoter region of the H-PGDS gene contains functional PPAR-response elements (PPREs). A synthetic PPARγ agonist can stimulate the up-regulation of H-PGDS in Se-deficient cells. Inhibition of H-PGDS activity blocks PPARγ-dependent gene expression. PPARγ function is likely mediated through the downregulation of NF-κB. Inhibition of NF-κB rescues H-PGDS expression in Se-deficient cells. Inhibition of NF-κB inhibits the NF-κB-dependent expression of mPGES-1 and simultaneously upregulates H-PGDS expression (Gandhi

et al., 2011).

Competition between H-PGDS and mPGES-1 has been previously reported. Supplementation of cells with mPGES-1 significantly increased the yield of PGE_2 and decreases the yield of PGD_2 (Yu et al., 2011). Similarly, when cells are supplemented with H-PGDS a significant increase is observed in the yield of PGD_2 and a corresponding decrease in the yield of PGE_2. When cells were treated with CAY10526 PGE_2 formation dropped to a basal level and PGD_2 formation increased. This effect can be attributed to the increased availability of PGH_2 to H-PGDS. In the absence of mPGES-1 activity more PGH_2 is shunted into the PGD_2 pathway. When H-PGDS is inhibited by HQL-79 an increase in PGE_2 formation is observed. Due to a lack of enzyme specificity with CAY10526 the redistribution effect observed with HQL-79 was much stronger (Yu et al., 2011).

15d-PGJ_2 can activate PPARγ and inhibit NF-κB independent of PPARγ. H-PGDS-dependent synthesis of 15d-PGJ_2 may create a positive feedback loop further increasing the expression of H-PGDS (Gandhi et al., 2011). Our data do not support positive regulation of H-PGDS by 15d-PGJ_2. H-PGDS expression was highest in HQL-79 and CAY10526 treated cells. Indomethacin induced greater expression of H-PGDS than in untreated cells. Elevated H-PGDS expression in the Se-supplemented uninhibited and CAY10526 samples would support positive regulation by 15d-PGJ_2. An upregulation of H-PGDS expression was observed only in response to Se-supplementation.

The upregulation of 15d-PGJ_2 synthesis is dependent upon the increased expression of H-PGDS which is in turn dependent upon the selenium status of the cell. By raising intracellular selenium levels to 250 nM maximum H-PGDS expression is achieved (Gandhi et al., 2011). In this study GPX1 expression was used as a measure of selenium status. The GPX family contains five isozymes; cytosolic GPX-1 is the most ubiquitously expressed. Under selenium deficient conditions selenoproteins are synthesized in a hierarchical order. GPX-1 is the first

selenoprotein to be downregulated in the event of Se-deficiency. This close link to selenium availability makes GPX1 a sensitive indicator of cellular selenium status (Lu and Holmgren, 2009). Negligible levels of GPX1 were expressed in Se-deficient cells; GPX1 expression was clearly observed in RAW 246.7 cells that were supplemented with 100 or 250 nM selenium. GPX1 expression plateaus at 100 nM selenium (Gandhi et al., 2011).

Supranutritional selenium doses are required for anti-carcinogenic effect. The average daily intake of selenium by US residents is estimated at 70 to 100 μg (El-Bayoumy, 2001). The current recommended daily allowance (RDA) is only 55 μg/d (Schrauzer and Surai, 2009). Patients in the NPC trial were treated with 200 μg/d of selenium. Despite the doseage exceeding the RDA 4 fold, none of the patients demonstrated any signs of toxicity. The NPC trial clearly demonstrated that supplementation with 200 μg/d of selenium lowered total cancer incidence (Clark et al., 1996). Growing evidence supports that selenium intakes of 200 to 300 μg/d are optimal for the maintenance of health (Schrauzer and Surai, 2009). The upper safe limit for Se-supplementation is considered 400 μg/d (Zeng, 2009).

The RDA needs to be adjusted to reflect the beneficial effects of elevated selenium status. In RAW 246.7 cells Se-supplementation (250 nM) leads to a 3 fold increase in H-PGDS expression. Se-supplementation in mice was protective against FV-induced erythroleukemia. Further, research is required to quantify the impact of Se-supplementation on H-PGDS expression and downstream 15d-PGJ_2 synthesis in humans.

Conclusion

Apoptosis was only induced in LSCs that were treated with lipids from Se-supplemented cells. Apoptosis was inhibited if the synthesis of PGD_2 was blocked by inhibitors. We know that H-PGDS is upregulated in Se-supplemented samples. We know that upregulation of H-PGDS increases the synthesis of the terminal prostaglandin 15d-PGJ_2. We know that 15d-PGJ_2 can induce apoptosis in LSCs. The concentration of 15d-PGJ_2 was higher in lipid extracts from Se-supplemented cells. We can conclude that the observed induction of apoptosis by lipid extracts is due to the presence of 15d-PGJ_2.

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