ARTICLE TITLE: *Genistein Protects Hematopoietic Stem Cells against G-CSF-Induced DNA Damage*

SHORT TITLE: *Genistein limits DNA Damage*

Liliana R. Souza¹, Erica Silva¹, Elissa Calloway¹, Omer Kucuk¹, Michael Rossi², Morgan L. McLemore¹.

Authors and Affiliations:
1 - Winship Cancer Institute
Department of Hematology and Oncology, Emory University

2 - Winship Cancer Institute
Department of Radiology and Oncology, Emory University

Corresponding author:
Liliana R Souza
Winship Cancer Institute, Department of Hematology and Oncology, Emory University
1365C Clifton Rd, room C3078, Atlanta, GA, 30322
email: lsouza@emory.edu
Tel: 404-778-1829
Fax: 404-778-5520

CONFLICT OF INTEREST DISCLOSURES: All the authors have no conflict of interest to disclose.

GRANT SUPPORT: The current work was supported entirely by departmental funds. No governmental grants funded this research.

WORDS OF TEXT: 4,894

ABSTRACT: 199

FIGURES/TABLE: 6

REFERENCE COUNT: 50
Granulocyte colony-stimulating factor (G-CSF) has been utilized to treat neutropenia in various clinical settings. Although clearly beneficial, there are concerns that the chronic use of G-CSF in certain conditions increases the risk of myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML). The most striking example is in severe congenital neutropenia (SCN). SCN patients develop MDS/AML at a high rate that is directly correlated to the cumulative lifetime dosage of G-CSF. MDS and AML that arise in these settings are commonly associated with chromosomal deletions. We have demonstrated in this study that chronic G-CSF treatment in mice results in expansion of the hematopoietic stem cell population. In addition, primitive hematopoietic progenitors from G-CSF–treated mice show evidence of DNA damage as demonstrated by an increase in double strand breaks and recurrent chromosomal deletions. Concurrent treatment with genistein, a natural soy isoflavone, limits DNA damage in this population. The protective effect of genistein appears to be related to its preferential inhibition of G-CSF–induced proliferation of hematopoietic stem cells. Importantly, genistein does not impair G-CSF–induced proliferation of committed hematopoietic progenitors, nor diminish neutrophil production. The protective effect of genistein was accomplished with plasma levels that are attainable through dietary supplementation.
INTRODUCTION

Severe congenital neutropenia (SCN) is a rare, heritable disorder characterized by isolated neutropenia from birth (1). Prior to the clinical use of granulocyte colony-stimulating factor (G-CSF), individuals typically died before the age of 2 from overwhelming infections. With G-CSF treatment, SCN patients now routinely survive until adolescence or even adulthood. Unfortunately, a substantial number of SCN patients now develop myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML) (1, 2). After 10 years of G-CSF treatment, the rate of MDS/AML in SCN patients is estimated to be 2-3% per year (2). The etiology of AML in SCN is not well defined. The initial hypothesis was that a molecular defect responsible for SCN predisposed individuals to AML. Since accumulating evidence has demonstrated that MDS/AML arises from hematopoietic stem cells (HSC), this hypothesis appears less likely as the most frequently mutated gene in SCN, ELA2, is not expressed in HSCs. Another hypothesis is that chronic G-CSF treatment promotes expansion of a malignant myeloid clone. This remains a concern as the risk of MDS and/or AML roughly correlates with lifetime cumulative dosage of G-CSF (1, 3). Other researchers have suggested that G-CSF usage in certain settings may promote leukemic transformation (4-9). These studies are far from conclusive, as others have shown no increase in the risk of leukemic transformation and/or relapse with G-CSF therapy (10). In particular, a prospective study has shown that G-CSF usage did not increase the rate of relapse or decrease complete remission rates in AML (11). However, SCN is unique in its cumulative lifetime dosage of G-CSF. SCN patients typically receive G-CSF multiple
times per week for life, as opposed to patients undergoing chemotherapy who may receive a short term treatment.

G-CSF signals through the granulocyte colony-stimulating factor receptor (G-CSFR). The G-CSFR is a non-tyrosine kinase receptor that is present at low levels on HSCs (12). In mice, G-CSF treatment results in an increase in HSCs (13). It is possible that chronic G-CSF treatment results in HSC proliferation and acquisition of mutations. Several lines of evidence lend support to this hypothesis. First, acquisition of hyperproliferative mutations of the G-CSFR increases the risk of leukemic transformation (14). Second, it has been demonstrated that HSCs preferentially utilize error prone DNA repair pathways when entering cell cycle and chromosomal deletions are often seen not only in patients with SCN and AML, but also in patients with aplastic anemia treated with G-CSF (7, 9, 14, 15).

In this manuscript we provide evidence that prolonged G-CSF treatment results in genomic instability in murine HSCs. In addition, we demonstrate that treatment with the soy isoflavone genistein lessens DNA damage. This effect is achieved by utilizing genistein at a dosage that can be easily attainable by dietary supplementation, suggesting that genistein may be an effective preventive agent for those patients who require prolonged G-CSF treatment.
MATERIALS AND METHODS

Mouse strain:

C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All mice were housed in a specific pathogen-free environment. We used 6 to 10 week-old mice in all studies and the experiments were approved by the Emory University and Institutional Animal Care and Use Committee (IACUC protocol number: 2000678).

In vivo G-CSF treatment:

C57BL/6J mice were treated subcutaneously 5 times a week with G-CSF (10μg/Kg) (Neupogen, Amgen, Conejo Valley, CA, USA) or diluent alone (control mice) for different amounts of time varying up to 1 year.

Array-comparative genomic hybridization analysis:

Bone marrow cells were harvested from mice treated with G-CSF or diluent for 4 months. Lin-Sca+ cells were isolated using the instructions provided by the manufacturer (Easy Sep 18756, 19756A, Stem Cell Technologies). Genomic DNA was extracted from Lin-Sca+ cells using the Qiagen DNeasy kit, and quantification and quality assessment were performed with Picogreen, NanoDrop and a standard agarose gel. DNA was analyzed by array-comparative genomic hybridization (Array CGH) at the Florida State University NimbleGen Microarray Facility using NimbleGen 3×720K mouse whole-genome tiling arrays. Copy number abnormalities were identified using NimbleScan and BioDiscovery Nexus software. Aberrant segments were queried in the
UCSC Genome Browser (GRCh38/mm10) for overlapping BAC alignments to be used for FISH validation of copy number loss and gain. Data from the ACGH is available on the GEO repository under accession number GSE54737.

**Immunofluorescence microscopy**

Bone marrow cells from mice were harvested and LSK cells (lineage-negative, cKit+, Sca+) were sorted on a BD FACSARia (BD Biosciences, San Jose, CA, USA) and spotted on a slide. The cells were fixed using 10% methanol and 10% formalin in PBS and washed twice with PBS. Cells were then permeabilized, blocked and incubated with antibodies overnight at 4°C. The antibodies used individually were: pH2AX (07-164, Millipore), pGSK3 A555 (bs-5367R-A555, Bioss), cyclin D1 A555 (bs-0623R-A555, Bioss), cyclin D3 A555 (bs-0660R-A555, Bioss). The following day the sample was washed with PBS, incubated with a fluorescent secondary antibody when necessary (cat#A21429, Invitrogen) and DAPI stained (cat#D3571, Invitrogen). For quantification of immunohistochemistry, images from over 100 cells were captured using a Carl Zeiss LSM 510 META confocal microscope (Zeiss, Thronwood, NY, USA) with a Plan-Apo 63 × 11.4 oil immersion lens. The maximum intensity per nucleus was determined and background subtracted using Metamorph® software (Molecular Devices, Canada)
ROS analyses:

LSK cells were sorted, stained with DHeT (final concentration 50μg/ml) (cat# 7008, Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 20 minutes and analyzed by FACSCanto (BD Biosciences, San Jose, CA, USA).

Fluorescence in situ hybridization (FISH):

Lineage-negative Sca+ (Lin-Sca+) bone marrow cells were isolated, fixed in a 1:3 mixture of methanol: acetic acid and spotted onto a glass slide. For FISH analysis, the chromosome region-specific Bac clone for Abl1 (RP23-156H9) on mouse chromosome 2, and Tsc2 (RP23-438P15) on mouse chromosome 17, were labeled with 5-ROX dUTP by nick translation (Empire Genomics, Buffalo, NY, USA). The denaturation, hybridization, and signal detection procedures were carried out as described by the Oncology Cytogenetics Facility at Emory University. Hybridization was visualized on a LSM 510 META confocal microscope (Zeiss, Thornwood, NY, USA).

In vivo genistein treatment:

C57BL/6J mice were treated subcutaneously 3 times a week for 6 weeks as follows: Genistein (10mg/kg) (Cayman Chemical Company, Ann Arbor, MI, USA) (genistein + 25 μl Peg400 + 75 μl 0.1% BSA in PBS ), G-CSF (10μg/Kg) (Neupogen, Amgen, Conejo Valley, CA, USA) (G-CSF + 25 μl Peg400 + 75 μl 0.1% BSA in PBS), Genistein + G-SCF (genistein + 25 μl Peg400 + G-CSF + 75 μl 0.1% BSA in PBS), Diluent (DMSO + 25 μl Peg400+ 75 μl 0.1% BSA in PBS ) (16, 17).
**Cell sorting and flow cytometric analysis:**

We used a BD FACSARia and FACSCanto (BD Biosciences, San Jose, CA, USA) for cell sorting and flow cytometric analysis, respectively, followed by analysis using FlowJo Software (Tree Star Inc). The following antibodies were used for cell sorting and flow cytometric analysis: MAC1 (cat#0112), GR1 (cat#5931), Ter119 (cat#5921), B220 (cat#0452), CD3 (cat#0031), c-Kit (cat#1171), Sca1 (cat#5981), CD48 (cat#0481), CD150 (cat#1501); all were obtained from eBiosciences.

**BrdU incorporation:**

To examine 5-bromo-2’-deoxyuridine (BrdU) incorporation we utilized the protocol previously described (18). Briefly, mice were given 3 daily intraperitoneal injections of BrdU in 0.1% BSA in PBS (Sigma, 3mg/24 hours) and maintained on 0.2mg/mL of BrdU in the drinking water for 72 hours. Mice were euthanized and bone marrow cells stained with antibodies against lineage markers, c-Kit, Sca, cd48, cd150. Cells were fixed, permeabilized and stained with anti-BrdU-PE (all from eBiosciences) and analyzed by FACSCanto.

**Statistical analysis:**

Data were analyzed with Microsoft Excel. Paired t tests were used to test for evidence of differences in groups. Values were considered statistically significant at P less than 0.05.
RESULTS

G-CSF treatment induces DNA damage in hematopoietic progenitors.

Mice were treated with G-CSF (10 µg/Kg 5 x week) for 4 months and bone marrow cells were analyzed by flow cytometry. The results demonstrated a 3-fold increase in the lineage-negative, Sca-positive and cKit-positive (LSK) population of G-CSF–treated mice (Figure 1A). As phosphorylation of histone H2AX (p-H2AX) is an indicator of DNA damage (19) we quantified the amount of nuclear pH2AX in individual LSKs using immunostaining. Our results demonstrate that there is a 2-fold increase in double strand breaks (DSB) in LSK cells of treated animals in comparison to control animals (Figure 1B, 1C). The LSK population was also analyzed for the presence of radical oxygen species (ROS). LSK cells from G-CSF–treated animals displayed a significant increase in ROS levels (Figure 1D).

G-CSF induces chromosomal instability

To determine if the increased proliferation and DNA damage induced by G-CSF treatment lead to chromosomal alterations, we performed array-comparative genomic hybridization analyses (aCGH). Bone marrow cells were harvested from three mice treated with G-CSF and three mice treated with diluent for 4 months. Genomic DNA from Lin-Sca+ cells of G-CSF treated mice and respective controls were analyzed in triplicate using NimbleGen 3×720K mouse whole-genome tiling arrays. Copy number abnormalities were identified using NimbleScan and BioDiscovery Nexus software. Aberrant segments were queried in the UCSC Genome Browser (GRCm38/mm10) for
overlapping BAC alignments to be used for fluorescent in situ hybridizations assay (FISH). Significant deletions in chromosome 2 and 17 were present in all G-CSF samples and were chosen for validation (Figure 2 A, B) (GEO accession number GSE54737).

Validation of the copy number abnormalities detected by CGH were examined using FISH analysis in Lin-Sca+ bone marrow cells from animals treated with G-CSF for 4 months using BAC clones RP23-156H9 (chromosome 2, Abl1) and RP23-438P15 (chromosome 17, Tsc2) within the regions of interest. Two sets of 100 cells per mouse (n=4) were counted and the average was calculated.

FISH analysis confirmed alterations in chromosome 2 in 17% of cells, compared to 2% in equivalent control mice (Figure 2C). Hybridizations also confirmed deletions in chromosome 17 in 31% of cells compared to 3% in equivalent control mice (Figure 2D). Greater than 90% of abnormal cells showed loss of one or 2 probe signals. In less than 10% of abnormal cells a gain of signal was noted.

**Genistein decreases G-CSF–induced DNA damage**

Previous studies have demonstrated that genistein protects hematopoietic progenitor cells from ionizing radiation and cytotoxic chemotherapy, therefore the following protocol was designed to examine whether genistein could protect HSC against the deleterious effects of chronic G-CSF treatment.
Mice were concomitantly treated with G-CSF (10 µg/Kg 5 x week) and genistein 10 mg/Kg) subcutaneously 3 times a week for a total of 6 weeks. At 6 weeks of G-CSF treatment the maximum expansion of HSC was observed, with statistically significant evidence of genomic instability. The mean concentration of genistein in plasma reached 1.70 µmol/mL 24 hours after the last treatment. Mice were euthanized and bone marrow cells were evaluated by flow cytometry. The LSK cell population in the bone marrow increased 3.4-fold in mice treated with G-CSF, however, concurrent treatment with genistein lessened the expansion of LSK cells by 2.3-fold (Figure 3A, Supplemental Figure 1). The production of ROS increased after G-CSF treatment (Figure 1D); however, we found that the levels of intracellular ROS were significantly lower in mice treated simultaneously with genistein and G-CSF (Figure 3B). These cells were sorted and then analyzed for the amount of DNA double-strand breaks (DSB) via the presence of nuclear pH2AX. We found that LSKs from mice treated concurrently with genistein and G-CSF exhibited less DNA damage than LSKs from G-CSF–treated mice (Figure 3C).

**Genistein decreases chromosomal instability**

Since we determined that LSKs from mice treated concurrently with genistein and G-CSF had less DNA damage, we verified the copy number of aberrant genomic segments.

Lin-Sca+ cells were analyzed by FISH using BAC clones for chromosome 2 and chromosome 17. Two sets of 100 cells per mouse (n=4) were counted and the
average was calculated. Our results demonstrated that after 6 weeks of G-CSF treatment, Lin-Sca+ bone marrow cells have alterations in chr 2 (6 %) and chr 17 (20%) while cells from animals treated with genistein combined with G-CSF had fewer chromosomal abnormalities based on alterations in chr 2 (2%, Figure 3D) and chr 17 (9%, Figure 3E).

Bone marrow differentials showed that G-CSF increases the percentage of granulocyte and concomitant treatment with genistein did not interfere with granulocytes expansion (Figure 4A). Animals treated simultaneously with genistein and G-CSF had a similar level of total neutrophils as the animals treated only with G-CSF (Figure 4B). Therefore, our data suggest that genistein protects against the deleterious effects of G-CSF–induced excessive HSC proliferation and at the same time permits the desired increase in the neutrophil population.

**Genistein limits DNA damage through inhibition of proliferation**

To elucidate the mechanism of action of genistein we compared its effect to that of a known antioxidant, N-acetyl-cysteine (NAC)(20). Animals were treated with G-CSF for 5 days and with genistein or NAC (50 mg/kg) on the last 2 days of G-CSF treatment (Figure 5A, B, C). The expansion in the LSK population in mice treated with G-CSF or NAC + G-CSF was comparable. However, genistein inhibited the proliferative effect of G-CSF (Figure 5A). Our results also showed that while NAC + G-CSF–treated mice had significantly lower ROS levels than G-CSF–treated mice, short-term treatment with genistein (2 doses) had a modest effect on ROS concentration (Figure 5B). We
observed, however, that LSK cells treated with genistein and G-CSF had significantly less DNA damage while NAC showed no significant protective effect against DS breaks induced by G-CSF treatment (Figure 5C).

Next, we compared the proliferation of different bone marrow cell populations by analyzing BrdU incorporation in newly synthesized DNA (Figure 6A). Myeloid progenitors are found in the Lin-cKit+Sca- fraction while the LSK (Lin-c-kit+Sca+) cell surface markers identify a cell population highly enriched for hematopoietic stem cells (21, 22). There was no significant difference between BrdU+ incorporation in the myeloid progenitor population (Lin-cKit+Sca-) in mice treated with G-CSF or simultaneously treated with genistein and G-CSF (Figure 6B). However the percentage of BrdU+ cells in the LSK population was 70% in G-CSF–treated mice and 56% in mice concurrently treated with genistein and G-CSF (Figure 6C, Supplemental 2). These data suggest that genistein preferentially inhibits HSC proliferation while allowing proliferation/differentiation of myeloid progenitors.

To further elucidate the mechanism underlying this phenomenon, we investigated the effects of G-CSF-triggered myeloid differentiation on GSK3 phosphorylation and cyclin D1 and D3 expression in the presence of genistein.

GSK-3 regulates HSC self-renewal and lineage commitment (23, 24) and controls genes that are important for proliferation such as cyclin D1 (25) and D3 (26). Myeloid progenitor bone marrow cells from mice treated with G-CSF for 5 days and with
genistein on the last 2 days of G-CSF treatment were sorted, lysed and the proteins were analyzed by Western blot. Our results demonstrate that G-CSF treatment induces GSK3 phosphorylation and concomitantly cyclin D1 and D3 expression in comparison to untreated cells (Figure 6D). Genistein, however, blocked GSK3 phosphorylation and cyclin D1 and D3 induction in G-CSF treated mice. To evaluate the degree of G-CSF signal activation in LSK cells the levels of pGSK3 (Figure 6E), cyclin D1 (Figure 6F) and D3 (Figure 6G) were measured. LSK cells were sorted by flow cytometry directly on glass slides and analyzed by immunofluorescence. G-CSF treatment induces GSK3 phosphorylation and increases cyclin D1 and D3 levels (Figure 6 E,F,G). Genistein treatment inhibited G-CSF induced phosphorylation of GSK3, cyclins D1 and D3 in LSK cells.

Taken together these results suggest that genistein modulates G-CSF induction of the GSK3-cyclinD1/D3 pathway in LSK and myeloid progenitors; nonetheless, it does not interfere with myeloid proliferation and differentiation of neutrophils (Figures 4A, 4B).
DISCUSSION

The effects of prolonged G-CSF treatment on hematopoietic stem cells are not well understood. We hypothesized that excessive HSC proliferation induced by G-CSF could lead to deleterious consequences. Genistein is a soybean-derived isoflavone with antioxidant effects (27). It also has tyrosine kinase inhibitory properties that attenuate proliferation of both normal and cancerous cells (28). Based on these properties we hypothesized that genistein could counteract the deleterious effects of excessive HSC proliferation induced by G-CSF.

G-CSF is widely utilized in multiple clinical settings to lessen the effects of neutropenia. Although clearly beneficial, there are concerns about the long term effects of G-CSF. A particular concern is that G-CSF therapy may increase the risk of MDS and/or AML. G-CSF utilization in both aplastic anemia and Fanconi’s anemia has been associated with clonal evolution to AML (7, 9). Furthermore, usage of G-CSF has been associated with an increased risk of developing MDS/AML in women who undergo chemotherapy for breast cancer (29). However, use of G-CSF during treatment of AML in one large prospective study had no impact on complete response rate or relapse rate (11).

The most compelling evidence for the increased risk of MDS/AML through G-CSF therapy comes from SCN. While G-CSF clearly improves survival, there are several lines of evidence to suggest that G-CSF treatment contributes to the
development of leukemia in these patients. First, the risk of leukemia appears to correlate with the cumulative dose of G-CSF (3). Second, of all the congenital marrow failure syndromes predisposed to AML, SCN alone does not appear to be an HSC disorder. Since AML appears to rise from sequential mutations in HSC, this would suggest that therapy, not the intrinsic cell defect, is causal (30). It has been demonstrated that G-CSF does initiate signaling pathways in HSCs (31). In addition, the presence of hyperproliferative truncation mutations and an activating mutation of the G-CSFR have been associated with the development of AML in SCN (1).

This manuscript provides evidence that prolonged G-CSF exposure results in genomic instability in HSCs. After extended treatment with G-CSF there is a significant increase in DNA damage in LSK cells. HSCs from mice treated in vivo with G-CSF displayed consistent loss of regions of chromosome 2 and 17. Interestingly, the chromosome 2 deletions align with ABL1, which has previously been demonstrated to be involved in several rearrangements and chromosome translocations in various types of human leukemia (32). A mouse model of acute promyelocytic leukemia and a radiation-induced model of AML show deletions on chromosome 2 in a region containing the gene PU.1, a transcription factor critical for myeloid development (33). Previous reports have implicated heterozygous loss of PU.1 as contributing to the development of leukemia in the aforementioned settings and in other mouse models (34). Although similar deletions in PU.1 are rare in human AML, mutations in RUNX1, an important regulator of PU.1 expression, are commonly seen.
Consistent deletions on chromosome 17 that include the tumor suppressor gene TSC2 were also detected. TSC2 is a negative regulator of mTOR and recent studies have demonstrated that the expression of TSC2 is downregulated in Acute Leukemia patients (35). The mTOR pathway is frequently activated in blasts from AML (36) and high-risk MDS patients (37). Furthermore, constitutive activation of the AKT/mTOR pathway has been shown to induce acute leukemia in mice (38). Collectively, the results suggest that prolonged G-CSF treatment induces DNA damage in HSCs and genistein acts as a genoprotective agent in this setting.

Despite evidence of genomic instability in HSCs, none of the mice treated with G-CSF developed leukemia. Transgenic mice overexpressing G-CSF also do not develop leukemia (39). This is not unexpected, as patients with SCN only develop AML after years of G-CSF treatment and the prevalence even after 10 years of treatment is less than 50%. As expected, the number of HSCs at risk for developing a leukemogenic mutation is significantly higher in a human than in a mouse.

Although prolonged G-CSF exposure promotes genomic instability in HSCs and is associated with the development of AML, it remains the only effective treatment for SCN, besides a HSC transplant. An ideal treatment would promote late myeloid differentiation without affecting HSCs. An alternative strategy would be to co-administer a complex that selectively blocks the effect of G-CSF on HSCs. Based on previous studies, genistein is an attractive compound. Genistein is a natural soy
isoflavone with excellent bioavailability that has both anti-oxidant and anti-proliferative properties (16, 40, 41). Both oxidative stress and excessive proliferation have been postulated to result in genomic instability in HSCs. In reality, previous studies have demonstrated that genistein protects HSCs from radiation and chemotherapy (17, 42, 43).

G-CSF treatment leads to a modest increase in ROS in LSK cells, which is reduced by genistein (Figure 3B). Although oxidative stress has been implicated in genomic instability in HSCs, NAC treatment, while reducing ROS, did not reduce DNA damage. In addition, we did not observe increased 8-oxo-guanine levels in LSK cells treated with G-CSF (data not shown). Interestingly, ROS and cell cycle progression appear to be linked in HSCs and the reduction in ROS may merely reflect decreased proliferation (44).

In the current study, we utilized a dose of genistein that results in serum levels that can easily be obtained through oral supplementation (16, 45). At this dosage, genistein partially blocked the G-CSF–induced expansion of LSK cells and significantly reduced pH2AX levels in this population. This was also accompanied by a reduction in the level of cells with an abnormal FISH signal. Importantly, genistein did not block the G-CSF–driven expansion of mature neutrophils, as the total number of neutrophils in mice treated with G-CSF and genistein were the same as in mice treated with G-CSF alone (Figure 4A, B). Genistein appears to inhibit G-CSF–driven expansion of LSKcd48-
cd150+ cells (Supplemental Figure 2). This population, termed LSK-SLAM, is highly enriched for HSCs.

Genistein did not block G-CSF–induced expansion of a population enriched for myeloid progenitors (Lin-ScacKit+) (Figure 6B). Collectively, these results would suggest that the effects of genistein are mediated primarily through preferential inhibition of HSC proliferation, while not impairing myeloid progenitor proliferation and differentiation. Genistein inhibits G-CSF induced GSK3 phosphorylation and cyclin D1 and D3 induction in LSK and myeloid progenitors (Figure 6D-G). Our results suggest that there is a distinct requirement for GSK3/cyclin D1/D3 in G-CSF modulated pathways in myeloid progenitors and HSC.

It has been shown that the absence of GSK3β impairs long-term self-renewal capacity of HSCs, although it is not essential for myeloid development (23). While HSCs from the triple cyclin knock-out mouse (cyclins D1, D2, and D3) display delayed cell cycle entry and multilinage hematopoietic failure (46, 47) single knockouts for D1 or D2 display normal granulocyte counts. Mice lacking cyclin D3 show impaired neutrophil development, however, all D1-/−, D2-/−, D3-/− single knock-out mice present normal myelopoiesis (48).

The data imply that genistein treatment prevents G-CSF induced GSK3 phosphorylation thus activating GSK3. GSK3 has being shown to regulate cyclin D1 and D3 through various mechanisms including mRNA transcription, protein localization and
ubiquitin-dependent proteolysis (49), however the mechanism involved in genistein modulation of GSK3/cyclin D1/D3 in G-CSF-treated mice requires further elucidation.

Collectively, our results indicate that prolonged G-CSF treatment induces DNA damage in HSCs by initiating cell cycle progression. HSCs are long lived, quiescent cells that preferentially utilize non-homologous end joining (NHEJ) for DNA repair when progressing from G0 to G1 (19, 50). NHEJ is a relatively error prone DNA repair mechanism, and its preferential use by HSCs has been postulated as a reason that chromosomal deletions and translocations are often seen and are frequently causal in the development of acute leukemia (19). Further evidence is provided by recent whole genome wide array sequencing that has shown that HSCs accumulate mutations over time. Importantly, we demonstrate that genistein, at levels obtainable through dietary supplementation, is able to reduce DNA damage by attenuating G-CSF–induced HSC proliferation without compromising the ability of G-CSF to accelerate terminal neutrophilic differentiation. These results suggest that genistein may be an effective preventive agent in patients with SCN who require prolonged G-CSF support.
ACKNOWLEDGEMENTS

Dr. Omer Kucuk is a Georgia Cancer Coalition Distinguished Scholar. We gratefully acknowledge Dr. Debra F. Saxe and Faith Sheff (Emory University) for their assistance with the FISH assays. We thank Dr. Daniel R. Doerge (U.S. FDA, Jefferson, AR) for analyzing the total isoflavone levels on mice serum+. We thank Dr. Hanna J Khoury and Dr. Leon Bernal-Mizrachi (Emory University) for their critical reading of this manuscript. We thank Anthea Hammond (Emory University) for the proofing and editing of this manuscript. We thank Dr. Adam Marcus and Deborah Eltzroth Martinson at the Winship Cell Imaging Core, for their expertise on imaging acquisition. We thank Aaron Rae at the Flow Cytometry Core at Emory Children's Pediatric Research Center, for his assistance in cell sorting and analysis. Finally we thank Vinicius Miessler de Andrade Carvalho, PhD student at UNICAMP-Brazil, for his assistance on some of the assays using G-CSF treated and untreated mice during the summer of 2011.
REFERENCES

FIGURE LEGENDS

FIGURE 1: G-CSF induces proliferation, DNA damage and ROS production in LSK cells. C57BL/6J mice (n= 4/group) were given 5 doses/week of G-CSF or diluent for 4 months. Bone marrow LSK cells were isolated and analyzed by FACS Aria. A) Fold change in bone marrow LSK cells between both groups. Data represent the mean +/- SD of 4 mice/group, a = p<0.05. B) Fold change in DNA damage in sorted LSK cells quantified by intracellular staining of pH2AX, a = p<0.05. For quantification, images from over 100 cells were captured using a Carl Zeiss LSM 510 META confocal microscope (Zeiss, Thonwood, NY, USA) with a Plan-Apo 63 × 11.4 oil immersion lens. The maximum intensity per nucleus was determined and background subtracted using Metamorph© software (Molecular Devices, Canada). C) Immunofluorescence staining of pH2AX (red) in sorted LSK cells of G-CSF treated mice. Images were captured using a Carl Zeiss LSM 510 META confocal microscope with a Plan-Apo 63 × 11.4 oil immersion lens. D) Fold change in ROS levels measured by staining sorted bone marrow LSK cells with DHET, data represent the mean +/- SD of 4 mice/group, a = p<0.05.
FIGURE 2: G-CSF induces chromosomal abnormalities in progenitor cells. A, B) C57BL/6J mice (n=3) were given 5 doses/week of G-CSF or diluents (n=3) for 4 months. Lin-Sca+ cells were isolated from the bone marrow. DNA from 3 G-CSF treated mice and respective controls were analyzed by array-comparative genomic hybridization (Array CGH) at the Florida State University NimbleGen microarray facility using NimbleGen 3×720K mouse whole-genome tiling arrays. Copy number abnormalities were identified using NimbleScan and BioDiscovery Nexus software. Aberrant segments presented in G-CSF-treated mice in chromosomes 2 and 17 were queried in the UCSC Genome Browser (GRCm38/mm10) for overlapping BAC alignments to be used for FISH validation of copy number loss. (GEO accession number GSE54737) C, D) C57BL/6J mice (n=4) were given 5 doses/week of G-CSF or diluents (n=4) for 4 months. Lin-Sca+ cells from bone marrow were analyzed using FISH probes for mouse chromosomes 2 and 17. Data represent the mean +/- SD of abnormal cells per mouse, a = p<0.05.
FIGURE 3: Genistein protects LSK cells against G-CSF induced damage. C57BL/6J mice (n = 4/group) were concomitantly treated with G-CSF (10 μg/Kg 5 x week) and genistein (10 mg/Kg) subcutaneously 3 times a week for a total of 6 weeks.

A) Bone marrow LSK cells were isolated. Data represent the mean +/- SD in LSK fold change in each treatment group. B) Fold change in ROS levels measured by staining sorted bone marrow LSK cells with DHET. Data represent the mean +/- SD. C) Fold change in DNA damage in sorted LSK cells quantified by intracellular staining of pH2AX. Data represent the mean +/- SD. D, E) Bone marrow Lin-Sca+ cells were isolated. Hybridization patterns were analyzed using FISH probes for mouse chromosomes 2 and 17. Data represent the mean +/- SD of abnormal cells per mouse, a = p<0.05.
FIGURE 4: Genistein allows granulocytic differentiation. A) C57BL/6J mice (n = 4/group) were concomitantly treated with G-CSF (10 μg/Kg 5 x week) and genistein (10 mg/Kg) subcutaneously 3 times a week for a total of 6 weeks. Bone marrow cells were isolated and analyzed. Bone marrow cells were stained with Geimsa Wright Stain. Approximately 200 cells were counted and assessed for distribution of morphology. Myelocytes (Myel), Metamyelocytes (Meta), Promyelocytes (Pro). B) Average number of neutrophils per treatment group. Neutrophil number was acquired during the bone marrow differential cell count and multiplied by the total number of bone marrow cells, data represents the mean +/- SD.
FIGURE 5: Comparison of the effects of genistein and NAC on LSK cells treated with G-CSF. C57BL/6J mice (n = 3/group) were treated with 5 doses of G-CSF followed by 2 doses of genistein or NAC (50mg/kg). A single group was treated with diluent as a control. Bone marrow LSK cells were isolated by FACSARia. Data represent the mean +/- SD. A) Fold change in LSK cells in the 4 treatment groups. B) Fold change in ROS levels measured by staining sorted bone marrow LSK cells with DHET then analyzing, a=p<0.05 vs. diluent, b= p<0.05 vs. G-CSF. C) Fold change in DNA damage in sorted LSK cells quantified by intracellular staining of pH2AX. a=p<0.05 vs. diluent, b= p<0.05 vs. G-CSF.
FIGURE 6: Effect of genistein on HSC proliferation in mice treated with G-CSF.

C57BL/6J mice (n = 3/group, 4 groups) were given 3 intraperitoneal injections of BrdU (3 mg/24 hours) in PBS and maintained on 0.2 mg/mL of BrdU in the drinking water for 72 hours with concomitant injection of diluents, genistein, G-CSF or genistein + G-CSF. A) Gating strategy to analyze BrdU incorporation in mouse BM. B) Percentage of BrdU incorporation into myeloid progenitor Lin-Sca-cKit+ cells. Data represent the mean +/- SD. C) Percentage of BrdU incorporation into LSK cells. Data represent the mean +/- SD. a = p<0.05 vs. diluents; b = p<0.05 vs. G-CSF. D) C57BL/6J mice (n = 3/group) were treated with 5 doses of G-CSF followed by 2 doses of genistein. Progenitor cells were isolated and extracted proteins were analyzed by Western blot with antibodies against pGSK3β/α, cyclin D1, cyclin D3 and β-actin. Shown are representative results of one of three experiments. E, F, G) C57BL/6J mice (n = 3/group) were treated with 5 doses of G-CSF followed by 2 doses of genistein, LSK cells were FACS sorted in glass slides and stained against pGSK3β/α, cyclin D1, cyclin D3. Shown are representative results of one of two experiments. For quantification, images from over 100 cells were captured using a Carl Zeiss LSM 510 META confocal microscope (Zeiss, Thronwood, NY, USA) with a Plan-Apo 63 × 11.4 oil immersion lens. The maximum intensity was determined and background subtracted using Metamorph® software (Molecular Devices, Canada). Fold change in mean +/- SD of intensity.
Figure 2
Figure 6
Genistein Protects Hematopoietic Stem Cells against G-CSF-Induced DNA Damage

Liliana Souza, Erica Silva, Elissa Calloway, et al.

Cancer Prev Res  Published OnlineFirst March 10, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-13-0295

Supplementary Material
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2014/03/12/1940-6207.CAPR-13-0295.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/early/2014/03/08/1940-6207.CAPR-13-0295.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.