Fhit-Deficient Hematopoietic Stem Cells Survive Hydroquinone Exposure Carrying Precancerous Changes

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Abstract

The fragile FHIT gene is among the first targets of DNA damage in preneoplastic lesions, and recent studies have shown that Fhit protein is involved in surveillance of genome integrity and checkpoint response after genotoxin exposure. We now find that Fhit-deficient hematopoietic cells, exposed to the genotoxin hydroquinone, are resistant to the suppression of stem cell in vitro colony formation observed with wild-type (Wt) hematopoietic cells. In vivo–transplanted, hydroquinone-exposed, Fhit-deficient bone marrow cells also escaped the bone marrow suppression exhibited by Wt-transplanted bone marrow. Comparative immunohistochemical analyses of bone marrow transplants showed relative absence of Bax in Fhit-deficient bone marrow, suggesting insensitivity to apoptosis; assessment of DNA damage showed that occurrence of the oxidized base 8-hydroxyguanosine, a marker of DNA damage, was also reduced in Fhit-deficient bone marrow, as was production of intracellular reactive oxygen species. Treatment with the antioxidant N-acetyl-l-cysteine relieved hydroquinone-induced suppression of colony formation by Wt hematopoietic cells, suggesting that the decreased oxidative damage to Fhit-deficient cells, relative to Wt hematopoietic cells, accounts for the survival advantage of Fhit-deficient bone marrow. Homology-dependent recombination repair predominated in Fhit-deficient cells, although not error-free repair, as indicated by a higher incidence of 6-thioguanine–resistant colonies. Tissues of hydroquinone-exposed Fhit-deficient bone marrow–transplanted mice exhibited peneplastic alterations, including accumulation of histone H2AX-positive DNA damage. The results indicate that reduced oxidative stress, coupled with efficient but not error-free DNA damage repair, allows unscheduled long-term survival of genotoxin-exposed Fhit-deficient hematopoietic stem cells carrying deleterious mutations. [Cancer Res 2008;68(10):3662–70]

Introduction

The well-established course of chronic benzene toxicity, due to workplace exposure, includes decreases in circulating erythrocytes, leukocytes, and platelets and, if exposure continues, to pancytopenia, which may accompany bone marrow aplasia and myelodysplastic syndrome or development of acute myelogenous leukemia and lymphoma (1). Results of experiments in animals have shown that exposure to benzene leads to multisite carcinogenicity, with development of tumors of various glandular tissues, including mammary glands, ovary, and lung, as well as hematopoietic cells (2, 3). Benzene and its major metabolites are not mutagenic in the Ames Salmonella test (4) but induce chromosomal aberrations in vivo and in vitro (4–6). The major downstream benzene metabolites, hydroquinone and catechol, can probably be metabolically activated to the reactive species, semiquinones or quinones, which rapidly bind proteins or nucleotides as a result of the peroxidative action of bone marrow myeloperoxidase, or prostaglandin H synthase of bone marrow macrophages (1).

It has been reported that treatment with benzene metabolites, such as hydroquinone, can lead to increased intracellular production of reactive oxygen species (ROS) and ROS can induce or lead to DNA double-strand breaks that can be repaired through homologous recombination, which is not error-free (7). Increased DNA double-strand break levels can induce hyperrecombination, which can lead to deleterious genetic changes. Studies of the effects of catalase showed that increased homologous recombination due to exposure to hydroquinone or benzoquinone was abolished by the addition of an antioxidant, suggesting that increased homologous recombination mediates benzene-initiated toxicity and is consistent with a role for oxidative stress in this mechanism (7).

Although the DNA damage response checkpoint can be activated after exposure to benzene, whether the cell cycle progresses or is suppressed has been the subject of controversy. Comparative gene expression profiling of p53 knockout and wild-type (Wt) mice has revealed altered expression of cell cycle, apoptosis, and growth control genes in hematopoietic samples from benzene-exposed mice, indicating that benzene exposure activates checkpoint proteins such as p21 and p53 (8, 9), which inhibit DNA replication and apoptosis (10, 11). Recent studies indicate that p21 cooperates with Chk1 to prevent apoptosis during DNA replication fork stress, which is important for maintaining chromosome stability (11). How chromosomal aberrations, which characterize tumor cells, are induced after exposure to benzene metabolites remains to be elucidated.

Common chromosome fragile sites are large regions that preferentially exhibit gaps or breaks, visible in metaphase chromosomes, when cells are exposed to replicative stress conditions and DNA synthesis is perturbed (12). FRA3B and FRA16D, at chromosome regions 3p14.2 and 16q23.3, are the two most active or most fragile of the common human fragile sites and are frequently deleted or altered in environmental carcinogen-associated cancers and in hematopoietic disorders (12, 13).
Genomic sequences at fragile sites (14, 15) and protein signal pathways that monitor their stability, including Atr (16), Brcal (17), Smc1 (18), Fanconi anemia pathway proteins (19), Chk1 (20), Hus1 (21), and Rad51 (22), have been characterized. Homology-dependent recombination (HRR) and nonhomologous end-joining (NHEJ) repair pathways regulate FRA3B and FRA16D fragile site stability, indicating that double-strand breaks are formed at common fragile sites as a result of replication perturbation (22). It is proposed that the fragile sites are so-called replication difficult zones, susceptible to delay of DNA replication, which would be monitored by DNA damage response checkpoints (23).

Paradoxically, the FHIT gene product, Fhit, is involved in maintenance of genome integrity at the mid-S checkpoint through the Atr/Chk1 pathway, and Fhit deficiency alters the response to DNA-damaging agents (24–26). The introduction of exogenous Fhit into cells in vitro led to modulation of expression of checkpoint proteins Hus1 and Chk1, at mid-S checkpoint, modulation that led to induction of apoptosis in esophageal cancer cells but not in noncancerous primary cells (27). However, the biological significance of Fhit deficiency in environmental carcinogen-related hematopoietic disorders had not been investigated.

Here, we report that absence of Fhit in hydroquinone-exposed mouse hematopoietic cells led to resistance to induction of cell death in vitro and escape from bone marrow suppression in transplanted mice. Immunohistochemical analyses of transplanted hydroquinone-exposed, Fhit knockout (Fko) and WT bone marrow revealed relative absence of apoptosis and senescence markers in Fko bone marrow, in parallel with reduced detection of the oxidized base, 8-hydroxyguanosine (8-OHdG); treatment with the antioxidant N-acetyl-L-cysteine (NAC) alleviated the hydroquinone-induced suppression of colony formation by WT hematopoietic cells. Accordingly, the long-term survival of hydroquinone-exposed Fhit-deficient bone marrow–transplanted mice allowed accumulation of in accurately repaired DNA lesions and premalignant alterations in bone marrow–derived cells, suggesting that Fhit deficiency leads to unscheduled survival of genotoxin-exposed bone marrow cells, allowing increase of stem or precursor cells with damaged genomes and resultant accumulation of genomic alterations.

Materials and Methods

Cell culture. Cells were cultured in DMEM supplemented with 10% FBS (10% DMEM). Mouse embryo fibroblasts (MEF) were separated from s.c. tissues of day 13.5 Fhit−/− embryos. Cell viability and death were assessed by direct visualization of cell morphology, trypan blue exclusion, Hoechst 33342 vital staining, and flow-assisted cytometric analysis of cells with sub-G1 DNA content, methods which showed good general agreement. All experiments involving animals were carried out in accordance with the guidelines of the institutional animal use committee under approved protocols. For hydroquinone exposure, cells were cultured in medium with 100 μmol/L hydroquinone (Quinhydrone, Sigma-Aldrich) at 37°C for indicated period, washed twice, and subjected to assay. Quinhydrone is a mixture of hydroquinone and benzoquinone, both metabolically active benzeno derivatives.

Flow cytometry. The following monoclonal antibodies (mAbs) were used for flow cytometric analysis: mAbs against Kit, Sca-1, CD4, CD8, B220, Gr-1, CD34, and Thy1. All mAbs were purchased from BD Biosciences. Cells were probed with antibodies to lineage markers (CD4, CD8, B220, Gr-1, CD34, and Thy1), collected by streptavidin-coated magnetic beads, and separated by automated magnetic cell sorting (MACS; Miltenyi Biotec) for the positive and negative cells for the lineage markers. After negative selection by the lineage negative markers (CD4, CD8, B220, Gr-1, CD34, and Thy1), cells were selected for Kit+, Sca-1−, and Lin− by using an Aria multicolor sorting system (Becton Dickinson). Cell cycle analysis was performed using a FACScan multicolor detection system (Becton Dickinson) after ethanol fixation and propidium iodide staining.

In vitro hematopoietic colony formation assays. Hematopoietic cells from murine bone marrow were obtained by dissection and washing out of long bones with PBS; marrow was passed through a 74-μm nylon mesh (Corning) to obtain single-cell suspensions. After staining with Turk solution, nucleated cells were counted using a hemacytometer and plated in triplicate in Iscove-based methylcellulose medium supplemented with erythropoietin, stem cell factor, interleukin (IL)-3, IL-6, insulin, and transferrin (MethoCult M3434, Stem Cell Technologies) with or without hydroquinone exposure for 5 h. Erythroid [colony-forming unit (CFU-E) and blast-forming unit (BFU-E)] hematopoietic progenitors were scored by morphologic criteria at days 3 and 5, respectively. Myeloid [granulocyte-macrophage CFU (CFU-GM), granulocyte CFU (CFU-G), and macrophage CFU (CFU-M)] and multilineage (CFU-Mix) hematopoietic progenitors were scored at day 7. Statistical significance was determined using nonparametric Kruskal-Wallis tests and Scheffe’s. For assessment of the effect of the antioxidant NAC (Sigma-Aldrich), Kit+/Sca-1−/Lin− (KSL) cells from bone marrow were cultured on OP9 stroma cells with or without 100 μmol/L NAC. After 4 wk, colonies were counted under a microscope (28).

Peripheral hematopoietic cells. Peripheral blood was collected from tails of heparinized tubes. After counting, erythrocytes were hemolyzed and the number of leukocytes and platelets was counted. The histogram of leukocytes was determined by Wright-Giemsa staining.

Assessment of ROS. ROS was measured by the Image-IT LIVE Green ROS Detection kit (Molecular Probes) according to the company’s instruction. Briefly, WT and Fko MEF were grown in coverslips immersed in six-well plates in 10% DMEM overnight, incubated in medium with or without 50 or 100 μmol/L of hydroquinone for 5 h, and grown overnight. Cells were labeled with 25 μmol/L carboxyl-H2 DCFDA [5-(and-6)-carboxy-2′,7′-dichlorodihydro-fluorescein diacetate]. After counterstaining with Hoechst 33342, cells were washed, mounted in warm water, and imaged immediately.

For hematopoietic cells, WT and Fko mice were sacrificed to obtain bone marrow cells, which were sorted by the MACS system of lineage marker antibodies, and 5 × 105 cells were incubated and treated by hydroquinone as above in six-well plates and subjected to fluorescence-activated cell sorting (FACS) analysis.

Real-time reverse transcription-PCR assays. Cells were isolated in cold PBS, and total cellular RNAs were isolated with an RNeasy kit (Qiagen). Total RNA (1 μg) was used for Taqman reverse-transcription (Applied Biosystems), real-time PCR was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems) using Taqman Universal PCR master mix and Taqman gene expression assays (Applied Biosystems). For granulocyte-specific gene expression, real-time PCR for granulocyte colony-stimulating factor (G-CSF) receptor, lysozyme M, neutrophil gelatinase-associated lipocalin precursor (NGAL), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed by a Taqman assay on an ABI 7000 system. PCR primers and probes were designed as follows (29): murine G-CSF receptor, 5′-CTAACATCTCCTCTTTAGCTT-3′ (sense), 5′-GGCATGGAGTTGAGATGATGATG-3′ (antisense), and 5′-AGTGCACATGGTCTGGCAGT-3′ (antisense); murine NGAL, 5′-GGGCTACAAGGGGACCACCA-3′ (sense), 5′-TCCGTCAGGCTATCAGT-3′ (antisense), and 5′-AGTGCACATGGTCTGGCAGT-3′ (antisense); murine NGAL, 5′-GCGCTACAAGGGGACCACCA-3′ (sense), 5′-TCCGTCAGGCTATCAGT-3′ (antisense), and 5′-AGTGCACATGGTCTGGCAGT-3′ (antisense); and murine GAPDH, 5′-ACGCGAATTTCAACGGCA-3′ (sense), 5′-AGATGGT-3′ (antisense), and 5′-AGAAGCGCAATGG-3′ (antisense).
performed in a 50-μL volume containing 100 mmol/L of appropriate detection probe. After normalization by the hypoxanthine guanine phosphoribosyltransferase (HPRT) and GAPDH expression levels, relative expression values of each experiment were calculated by defining the mean value for control as 100%.

Assessment of repair activity. NHEJ and HR were assessed in cultured cells after transfection with the pBluescript vector, as described (30), and specific HR activity was assessed using the I-SceI system, as described (31, 32).

Mouse bone marrow reconstitution. Fhit+/+ (WT) and Fhit−/− (Fko) mice on C57BL/6 (B6-Ly5.2) background were used. C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.1) were obtained from Sankeo Lab Service. Lethally irradiated (9.5 Gy) C57/BL/6-Ly5.1 congenic mice were reconstituted with hydroquinone-treated or untreated mononuclear cells from bone marrow of Fhit−/− or Fhit+/+(C57/BL/6-Ly5.2) mice. Reconstitution of donor (Ly5.2) myeloid and lymphoid cells was monitored by staining blood cells with antibodies against Ly5.1, Ly5.2, CD3, B220, Mac-1, and Gr-1.

Protein analysis. For immunoblot analysis, 5 × 106 cells in six-well plates were grown overnight. Cells were lysed in lysis buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 2% NP40, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L glycophosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1 μg/ml leupeptin) for 30 min and centrifuged at 10,000 × g at 4 °C for 20 min, and the soluble fraction was used for the experiment. After measuring the concentration of protein using a Bio-Rad kit, 10 μg protein was separated in 4% to 20% gradient SDS-PAGE for 2 h at 4 °C, transferred to membranes, and probed with primary antibodies, which were detected with secondary antisera in the enhanced chemiluminescence system (Amersham).

Comet assay. To detect repair activity in situ, the comet assay system was applied according to the manufacturer’s instruction (Trevigen). Briefly, bone marrow–derived cells were exposed to hydroquinone in vitro for 5 h followed by culture in the fresh medium for 2 h and embedding in low-melting agarose on glass slide. After treatment with lysis and alkaline solution, electrophoresis was applied to the slide in 1 × Tris-borate EDTA buffer. Slides were dipped in 70% ethanol, air dried, fixed in acetic acid and methanol, and silver stained, and the reaction was stopped in 5% acetic acid. The slides were microscopically examined and 2,000 cells were scored per sample.

Assessment of mutation frequency in MEFs. The frequency of hydroquinone-induced mutations at the HPRT locus was assessed. MEFs were grown in DMEM containing HAT supplement (100 μmol/L hypoxanthine, 0.4 μmol/L aminopterin, and 16 μmol/L thymidine; Life Technologies) for 10 d to reduce the number of preexisting 6-thioguanine (6-TG)-resistant, HPRT-deficient mutants. To study hydroquinone-induced mutagenesis, 2 × 106 Fko and Wt cells were seeded in each of five 150-mm dishes overnight, then cultured in medium with 50 μmol/L hydroquinone for 6 h, and washed twice with fresh growth medium, and the medium was replaced with medium containing 20 μmol/L 6-TG. Two days later, all plates were trypsinized, cells were resuspended in one tube for Fko and one for Wt, and 2 × 104 cells were replated in each of four 100-mm dishes in 20 μmol/L 6-TG (Sigma-Aldrich) for both cell types. After 14 d, colonies (>50 cells) were counted and the frequency of 6-TG–resistant mutants per initial plating, corrected for plating efficiency for each cell type, was calculated. Average plating efficiency for Fko cells was 95.3% and for Wt was 96.5%.

Histological analysis of bone marrow sections. After decalcification and antigen retrieval, endogenous peroxidase was inhibited with 3% hydrogen peroxide, and nonspecific binding sites were blocked with normal goat serum. Slides were incubated overnight with anti-CxR against Bax (Santa Cruz Biotechnology), p38 (Santa Cruz Biotechnology), phospho-p38 (Cell Signaling), phospho-Chk1 (Cellbiochem), CD3 (BioLegend), CD11b (AbD Serotec), or CD45 (BioLegend) at 1:500 followed by incubation with biotinylated secondary antibody. Slides then were incubated with streptavidin horseradish peroxidase (1:1,000; Dako). For assessment of apoptotic index, apoptotic cells were stained using the in situ oligo ligation apoptosis (Chemicon) detection system. Histological analysis was performed for each recipient mouse sacrificed by evaluation of at least 10 low-power field areas (magnification, ×100).

Detection of 8-OHdG in mouse bone marrow tissue. Mouse bone marrow tissues, 60 and 120 d after transplant, were embedded in paraffin and consecutive 4-μm tissue sections were cut from paraffin blocks and placed on polylysine-coated slides for immunofluorescence analysis. Slides were deparaffinized, and endogenous peroxidase was blocked by incubation in 3% H2O2 for 30 min at room temperature. Slides were treated with proteinase K (10 μg/ml in PBS (pH 7.4)) at 37 °C for 40 min before blocking with goat IgG serum for 30 min at room temperature. Then, slides were incubated with goat anti-8-hydroxy-2′-deoxyguanosine and 8-OHdG polyclonal antibody (Chemicon) for 60 min at 37 °C (×200). Negative control slides were prepared by replacing primary antibodies with PBS, Texas red–conjugated anti-goat serum (Santa Cruz Biotechnology) was used as secondary antibody (1:100). Slides were examined using a fluorescence microscope.

Statistical analyses. For statistical analysis of significance of differences in production of ROS and other biochemical features in Wt and Fko MEFs after hydroquinone treatment, both one- and two-tailed Student’s t test was done. P values of <0.05 were considered to be statistically significant. Error bars represent SE.

Results and Discussion

Hydroquinone-induced suppression of hematopoietic colony formation. The effects of hydroquinone exposure on Fko and Wt bone marrow cells were assessed by in vitro colony formation assays (Fig. 1). To assess the effect of hydroquinone exposure on hematopoietic stem cells (HSC), the KSL fractions from Fko and Wt hematopoietic bone marrow cells were isolated (Fig. 1A) and in vitro colony formation potential was assessed (Fig. 1B). Unexposed Fko and Wt bone marrow mononuclear cells showed similar BFU-E, CFU-M, CFU-G, and CFU-GM colony-forming efficiency (Fig. 1C, bottom). In addition, the appearance and expression of differentiation-specific markers by Fko and Wt committed hematopoietic colonies (Fig. 1B and C, top) did not differ significantly in the absence of hydroquinone. The number of CFU-M and CFU-G colonies formed by Wt KSL cells was reduced relative to Fko KSL cells (<0.05) but BFU-E colony numbers did not differ significantly (Fig. 1D, top). We next used Lin− bone marrow cells for in vitro colony formation assays (Fig. 1D, bottom). The results were similar to those of KSL fractions; that is, CFU-M and CFU-G colony numbers from Wt Lin− cells were reduced relative to Fko Lin− bone marrow cells, a difference not observed without hydroquinone. The results suggest that Fhit plays a role in response to hydroquinone-induced inhibition of hematopoietic colony formation potential, with Fhit deficiency allowing resistance to genotoxic stress-induced suppression of hematopoietic colony formation.

Fhit-deficient bone marrow is resistant to genotoxin-induced suppression in vivo. Mononuclear cells of Fko and Wt bone marrow, with or without hydroquinone exposure, were transplanted to Wt mice previously exposed to 9.5 Gy ionizing irradiation (Fig. 2A, top). Hematopoietic cell counts in peripheral blood showed that neutrophils, lymphocytes, monocytes, basophils, eosinophils, and platelets increased gradually between 4 and 42 days after transplantation of both Fko and Wt cells (Supplementary Fig. S1), indicating that short-term progenitors are involved in increase and maintenance of Fko and Wt hematopoietic cells in the relatively early posttransplantation phase. Mice receiving non–hydroquinone-exposed Fko or Wt bone marrow cells all survived to 120 days after transplantation (data not shown). In sharp contrast, the recipient mice showed ~80% lethality by 120 days after transplantation of hydroquinone-exposed Wt bone marrow cells; hydroquinone-induced lethality
was only ~25% by day 120 in Fko bone marrow recipients (Fig. 2A, bottom), showing that Fhit deficiency abrogates genotoxic-induced suppression of hematopoiesis.

Because the hydroquinone-treated bone marrow transplantation experiments resulted in reduced survival of recipient mice, competitive transplantation assays were performed; untreated or hydroquinone-treated Fko or Wt HSCs were mixed with congenic Ly5.1 mouse bone marrow cells, and the mixtures were transplanted into irradiated mice of Ly5.1 background. The Ly5.1 bone marrow cells would support survival of recipient mice so that the contribution of donor hydroquinone-treated HSCs could be evaluated in the transplant recipient chimeric mice (33, 34). The sorted KSL fractions from Fko and Wt mice were exposed to hydroquinone in vitro, mixed with bone marrow cells from Ly5.1 mice, and transplanted to Ly5.1 recipient mice. All the recipient mice, two receiving Wt and three receiving Fko bone marrow, survived.

Assessment of hydroquinone-exposed donor cells in the Ly5.1 background showed that Fko bone marrow cells contributed to increased chimerism (Fig. 2D, right). Flow cytometer analysis of peripheral mononuclear cells indicated that Kit+ and Sca-1+ cells were increased in mice receiving hydroquinone-exposed Fko donor marrow compared with hydroquinone-exposed Wt marrow (4.6–8.3 times increase in Kit+ Fko compared with Kit+ Wt; ~3.5 times increase in Sca-1+ cells; Fig. 2C, representing two recipients of Wt and two of Fko bone marrow). Even in the absence of hydroquinone exposure, Kit+Sca-1+ cells were increased in mice receiving Fko donor marrow compared with Wt marrow (3.2 times in Kit+; ~1.17 times in Sca-1+ cells; Fig. 2B). The fraction of Kit+,
Sca-1⁺, and Lin⁻ cells play a significant role in self-renewal and multipotent differentiation of HSCs (33). A serum-free single-cell culture followed by transplantation of cultured cells into lethally irradiated mice indicated that Kit⁺, Sca-1⁺, Lin⁻ cells are highly enriched for murine bone marrow HSCs (34). The above results suggest that (a) Fhit-deficient mice maintain a larger fraction of Kit⁺, Sca-1⁺ cells than Wt mice, as apparent after transplantation of hydroquinone-exposed marrow, and (b) Fhit-deficient cells preserve or retain potential for self-renewal and multipotent differentiation after genotoxic stress.

Genotoxic-induced DNA damage and cell death are inhibited in Fhit-deficient marrow. Histologic assessment of bone marrow was performed by sacrificing recipient mice ~120 days after transplantation of hydroquinone-exposed nucleated cells (Fig. 3A). H&E staining of Wt bone marrow showed gross reduction of nucleated bone marrow cells, compatible with the preclinical stage of aplastic anemia and likely related to cause of death of recipient mice. Immunohistochemical analysis of bone marrow after transplantation of hydroquinone-exposed nucleated cells showed patchy positive staining of Bax and phosho-p38 throughout Wt bone marrow, indicating induction of apoptosis and senescence, whereas Fko bone marrow showed relatively healthy cellularity with less Bax and phosho-p38 expression (Fig. 3A). The assessment of apoptotic index showed efficient induction of apoptosis in transplanted bone marrow of hydroquinone-exposed Wt cells but much less apoptosis in Fko bone marrow (Fig. 3B and C).

Assessment of DNA damage by detection of 8-OHdG in genotoxic-exposed Fko and Wt hematopoietic cells showed that 8-OHdG was reduced in Fhit-deficient bone marrow cells, indicating that reduced oxidative stress is associated with enhanced survival of hydroquinone-exposed Fko bone marrow (Fig. 4A). We then assessed the effect of oxidative stress on survival of HSCs in vitro. As expected, hydroquinone exposure resulted in a relatively greater reduction of Wt colonies (Fig. 4B). Treatment with NAC antioxidant resulted in significant recovery of colony-forming ability of Wt HSCs and, to a lesser extent, of Fko stem cells in long-term in vitro culture, suggesting that treatment with NAC or other antioxidant might prolong the survival of mice receiving hydroquinone-treated Wt bone marrow. The recovery of colony formation by NAC treatment indicates that hydroquinone-induced genotoxic stress, followed by apoptosis, is involved in suppression of colony formation by hematopoietic stem or precursor cells, a process subverted by Fhit deficiency. The data show that Wt stem cell colony-forming ability was suppressed by hydroquinone, likely due to an increase in ROS by hydroquinone treatment (35, 36), which causes DNA damage and apoptosis, whereas in the absence of Fhit ROS-induced DNA damage and apoptosis was reduced, leading to colony growth and mouse survival. This experiment accords with the recent demonstration that Fhit interacts with the ferredoxin reductase (Fdxr) protein (37), a 54-kDa mitochondrial flavoprotein responsible for transferring electrons from NADPH to cytochrome P450 via ferredoxin. Leakage of electrons from this shuttling system can result in generation of ROS (38). Following application of oxidative stress, the Fhit-Fdxr interaction leads to ROS generation, an early event in Fhit-triggered apoptosis. In similarly treated Fhit-deficient cells, much less ROS is generated, allowing unscheduled survival of damaged cells (37), a mechanism with relevance to early events in carcinogenesis and to refractoriness to chemotherapy of Fhit-deficient cells.

To confirm that hydroquinone treatment leads to decreased ROS production in Fko cells relative to Wt cells, both MEFs and bone marrow cells were treated with 50 or 100 μmol/L of hydroquinone; ROS production was assessed by FACS analysis after staining of bone marrow–derived cells with DCFDA and by fluorescence microscopy and positive cell counting after staining of MEFs with DCFDA. The results of the FACS analysis of the sorted bone marrow cells are shown in Supplementary Fig. S2 and illustrate that, after treatment

**Figure 2.** Bone marrow transplantation. Nucleated bone marrow cells (5 × 10⁶) from Fko or Wt mice were transplanted into 9.5 Gy–irradiated Wt mice. A, top, experimental design; bottom, survival of transplanted mice. Bone marrow (BM) cells with (bottom) or without exposure (data not shown) to hydroquinone were transplanted. All recipients of non–hydroquinone-treated bone marrow survived (data not shown), whereas recipients of hydroquinone-treated Fko bone marrow showed a 3-fold survival advantage over hydroquinone-treated Wt recipients at 120 d. B to D, competitive transplantation assays were also performed. B, non–hydroquinone-treated peripheral nucleated cells were analyzed for Kit and Sca-1 positivity by flow cytometry. C, hydroquinone-exposed hematopoietic fraction of bone marrow cells (2 × 10⁷ KSL) from Fko or Wt mice were mixed with 3 × 10⁶ bone marrow cells from congenic Ly5.1 mice and transplanted into Wt Ly5.1 mice. After ~70 d, Ly5.2-positive peripheral nucleated cells were collected by MACS and analyzed for Kit and Sca-1 positivity by flow cytometry. D, left, experimental design; right, chimerism was assessed by the presence of the Ly5.2-positive fraction of hydroquinone-exposed Fko or Wt hematopoietic cells.
with 100 μmol/L hydroquinone, the Wt hematopoietic cells showed >2-fold more ROS production than Fko hematopoietic cells.

For the MEF cells, the fraction of Hoechst-positive cells that showed ROS fluorescence (examples shown in Supplementary Fig. S3A) were counted under confocal microscope and the quantitative estimates of ROS-positive fractions of Wt and Fko MEFs are shown in Supplementary Fig. S3B. The results indicate that 2-fold more of the Wt MEFs than Fko MEFs produced measurable ROS after 50 μmol/L hydroquinone (P < 0.022) and 100 μmol/L hydroquinone (P < 0.001) treatment.

**Repair pathways in Fhit-deficient bone marrow cells.** Recent studies have indicated that Fhit is involved in maintenance of genome integrity at the mid-S checkpoint and that Fhit deficiency alters the response to DNA-damaging agents (24–27). We have thus examined the efficiency of DNA damage repair by comparative assessment of the frequency of usage of the two pathways, HRR and NHEJ, in Wt and Fko cells. By combining a I-SceI–induced DNA double-strand break system and small interfering RNA approach, it was previously shown that knocking down Fhit increased the efficiency of HRR in cells, whereas knocking down Chk1 decreased the efficiency of HRR, and NHEJ was independent of Fhit status (26). To assess the effect of hydroquinone on DNA repair in hematopoietic cells, pRec1 reporter plasmids were introduced into Fko and Wt bone marrow cells, and cells were cultured in medium with hydroquinone to induce DNA damage and repair activity; plasmid DNA was recovered and sequenced (Fig. 5A, top) to determine repair pathways used; plasmids with small deleted regions (Fig. 5A, middle) were considered evidence of similar levels of NHEJ in both cell types. Clones from Fko cells, but not Wt cells, with relatively large deletions within CDKN2 genomic sequences, representing HRR, were also sequenced (Fig. 5A, bottom). The ratios of sequenced clones derived from HRR versus NHEJ pathways indicated that HRR occurred more frequently than NHEJ in Fko cells (Fig. 5B); the reverse was observed in Wt cells. To further assess the repair activity in cells in situ, comet assays were performed using hydroquinone-exposed Fko and Wt marrow cells. In this assay, cells in situ are lysed and exposed to alkali and an electrophoretic field, in which damaged DNA fragments would migrate out of the cell, appearing as “comet” signs when assessed, whereas undamaged or repaired DNA would migrate slower and...
remain within the confines of the nucleoid when current is applied. On microscopic examination, characteristic comet tails were apparent in Wt cells 3 to 6 h after hydroquinone exposure, whereas comet tails were less frequent in Fko cells (Supplementary Fig. S4A and B), suggesting that DNA repair was occurring more actively in Fko cells; this conclusion is consistent with the above finding that Fhit deficiency leads to resistance to genotoxin-induced cell elimination through apoptosis.

**Mutation in hydroquinone-exposed MEF cultures.** To determine if hydroquinone exposure differentially affects the occurrence of mutations in Fhit-positive and Fhit-negative cells, hydroquinone-treated MEFs from Wt and Fko mice were grown in 6-TG to allow growth and enumeration only of HPRT-deficient colonies; mutations in the *HPRT* gene allow growth of cells in the otherwise toxic 6-TG. MEFs were used because it would not have been possible to do this experiment with hematopoietic cells. In a preliminary experiment, we observed no difference in 6-TG surviving colonies in the two cell types without hydroquinone exposure, whereas 24 h of hydroquinone exposure caused apoptosis and significantly reduced cell viability in both cell types (data not shown). A 6-h treatment was chosen for the mutation frequency experiment (Fig. 5C). The data in Fig. 5C can be summarized as follows: after correction for plating efficiency, an average of 12.3 Fko colonies and an average of 7.9 Wt colonies per $1 \times 10^5$ cells seeded in 6-TG were observed, a significant difference ($P = 0.024$). A possible interpretation of these data is that more Fko than Wt cells survived the hydroquinone treatment and there was thus a larger population of surviving Fko cells that might carry mutation. That is, although ROS is reduced in Fhit-deficient MEF cells, there was hydroquinone-induced oxidative damage followed by DNA damage repair and overall reduction of apoptosis relative to Wt cells. An additional possibility is that repair in the Wt cells is more accurate. Although the Fko cells show a higher ratio of HRR to NHEJ repair than Wt cells, the repair is not error-free and the reduced apoptosis allows escape of cells with imperfectly repaired damage.

**Preneoplastic changes in hydroquinone-exposed Fko bone marrow recipients.** At day $\sim 200$ after transplantation, two of two surviving hydroquinone-treated Wt recipients and two of six surviving hydroquinone-treated Fko recipients were sacrificed and necropsied, and tissues were examinedgrossly, histologically, and immunohistochemically. The spleens and livers of the two Fko bone marrow recipient mice were grossly enlarged, and the sinusoid structure was reorganized in the spleen (Supplementary Fig. SS4)–C). Immunohistochemical staining of phospho-Akt and phospho-Chk1 indicated that positive cells were in the marginal area of spleen sinusoid of untreated Fko recipients, whereas the expression was reduced due to absence of sinusoid structures in the hydroquinone-treated Fko recipients (Supplementary Fig. SS5). The expression of histone H2AX was detectable in spleen of hydroquinone-treated Fko recipient compared with nontreated Fko (compare Supplementary Fig. SS6, a and b), suggesting increased DNA damage and checkpoint activation at 200 days (Supplementary Fig. SS6, b), as expected in a preneoplastic condition (39, 40). The staining of CD3, CD45, and CD11b indicated that lymphocytes are located in a less restricted structure in the spleen in the hydroquinone-treated Fko spleen at 200 days (Supplementary Fig. SS6, compare hydroquinone-treated with nontreated spleen sections), again suggesting premalignant changes.

In summary, this study has shown that reduced oxidative stress, coupled with active but imperfect DNA damage repair, allows unscheduled, long-term survival of genotoxin-exposed, Fhit-deficient HSCs, leading to accumulation of deleterious mutations. Fhit deficiency resulted in reduced ROS production in hematopoietic and MEF cells and consequent reduced apoptosis, as observed in epithelial cancer–derived cells lacking Fhit and thus lacking the Fhit-Fdxr complex involved in ROS production during oxidative stress (37). The survival advantage of Fhit-deficient cells during post-hydroquinone hemopoiesis is offset by the efficient but imperfect repair of DNA damage caused by severe oxidative stress, leading inevitably to further accumulation of genomic damage. The increased expression of H2AX, a marker of DNA double-strand breaks, from 120 to 200 days in spleens of hydroquinone-treated, Fko-transplanted mice suggests accumulation of DNA damage that could lead to frank malignancy.

As for the action of hydroquinone, previous comparative expression profiles illustrated partially overlapping signal pathways for total bone marrow and HSCs: checkpoint pathway genes were highly induced in actively proliferating bone marrow cells but were induced to a lesser extent in slowly proliferating HSCs following benzene exposure (41). Among reactions to hydroquinone exposure, we found that DNA damage repair activity and responsiveness to cell death were altered in Fhit-deficient cells, alterations that could contribute to an increase in genomic instability during hematopoiesis. Considering that the DNA damage checkpoint pathway can
induce immediate suppression of cell cycling of hemopoietic progenitors and prominent suppression of hemopoiesis, it has been proposed that the process of recovery from intermittent benzene-induced marrow suppression may be involved in benzene-induced leukemogenesis (8). Further study would include assessment of oscillatory changes in ROS, possibly associated with chromosome aberrations in hematopoietic disorders or in transplanted bone marrow cells, after exposure to genotoxin.

Disclosure of Potential Conflicts of Interest
K. Huebner: Coinventor on Fhit patent, licensed to Crogen. The other authors disclosed no potential conflicts of interest.
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