H-ferritin overexpression promotes radiation-induced leukemia/lymphoma in mice

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Introduction

Ferritin is a major iron storage protein expressed in cells and plays a key role in iron homeostasis (1). Mammalian ferritins are composed of 24 subunits that contain variable proportions of two subunits, H-ferritin (HF) and L-ferritin (LF). HF, a core subunit of ferritin, has ferroxidase activity and regulates iron-mediated oxidative stress by detoxifying Fe(2+) to Fe(3+) and performing the primary functions of ferritin. Although ferritin possesses many iron-related functions, recent studies have shown that it also has various functions seemingly unrelated to iron, such as regulation of apoptosis, protein translation and chemokine receptor signaling (1). Therefore, this protein is involved in a wide range of biological processes and in pathological conditions. Indeed, altered expression of ferritin is observed in some hematologic and neurodegenerative diseases in humans (2).

Abbreviations: BM, bone marrow; HF, H-ferritin; hHF, human HF gene; 8-OHdG, 8-hydroxydeoxyguanosine; LF, L-ferritin; PCNA, proliferating cell nuclear antigen; TdT, terminal deoxynucleotidyl transferase; tg, transgenic; TL, thymic lymphoma/leukemia; WT, wild-type.

Previous studies suggested ferritin may be involved in the pathogenesis of malignancies, such as elevated serum levels are used clinically as a biomarker for some kinds of cancer (2). The overexpression of ferritin was observed in various human tumors including colon cancer (3), breast cancer (4) and esophageal adenocarcinoma (5). HF was abundant in human mesothelioma cells (6) and controls tumor progression and metastasis via epithelial-mesenchymal transition (7). Of note, some previous investigations suggested that ferritin may be involved in the pathogenesis of hematologic malignancies. Elevated serum ferritin levels were associated with the overall survival of patients with multiple myeloma (8). HF was increased in immature and proliferative thymocytes and lymphoblastic lymphoma/leukemia relative to peripheral blood lymphocytes (9). HF is upregulated by the nuclear factor-kappaB signaling pathway (10), which is often constitutively active in many cancers, especially lymphomas (11,12). Recently, higher levels of HF were found in patients with myelodysplastic syndrome, a preleukemia condition with clonal growth of abnormal hematopoietic progenitors (13). These observations raise the possibility that HF may contribute to hematologic tumorigenesis rather than merely being a marker of malignant diseases. However, in vivo evidence that HF is directly linked to leukemia/lymphomagenesis has not yet been shown. This study aimed to determine the role of HF overexpression in leukemia/lymphomagenesis using radiation-induced thymic lymphomagenesis in mice, a classical model for the study of leukemia/lymphoma (14). For this purpose, we assessed the susceptibility of transgenic mice overexpressing human HF (hHF-tg mice) (15) to the development of thymic lymphoma/leukemia (TL). This study demonstrated that hHF-tg mice developed radiation-induced TL with a higher incidence and earlier onset than wild-type (WT) mice, providing in vivo evidence that HF overexpression is closely related to the development of leukemia/lymphoma.

Materials and methods

Mice

All animal experiments were approved by the Institutional Animal Care and Use Committee of Experimental Animals of the National Institute of Radiological Sciences. Generation of hHF-tg mice and genotyping were described previously (15). All hHF-tg mice used in this study were generated by crossing hHF-tg mice with C57BL/6J mice and thus were heterozygous for the transgene. Non-transgenic WT mice used as controls in this study were derived from littermates. Mice were bred and maintained in a specific pathogen-free animal facility with 12 h light and dark cycles at 22 ± 1°C and a relative humidity of 45–50%.

Western blot analysis

A detailed protocol for western blot analysis was described previously (6). Briefly, tissues and cells were lysed by T-PER (Thermo Scientific, Waltham, MA) and a mammalian cell lysis kit (Sigma–Aldrich, St Louis, MO), respectively. Lysates were separated on a 12.5% polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The primary antibodies used were as follows: anti-human HF rabbit polyclonal antibody (Ab) (H-53, sc-25617, Santa Cruz Biotechnology, Santa Cruz, CA); anti-human LF goat polyclonal Ab (D-18, sc-14420, Santa Cruz Biotechnology); anti-human actin goat polyclonal Ab (C-11, sc-1615, Santa Cruz Biotechnology). Secondary antibodies used were anti-rabbit IgG conjugated with horseradish peroxidase (NA934, GE Healthcare Bio Sciences, Little Chalfont, UK) or anti-goat IgG conjugated with horseradish peroxidase (sc-2020, Santa Cruz Biotechnology).

Radiation-induced tumorigenesis

Four-week-old mice were X-irradiated weekly with a 1.2 Gy fraction (total dose 4.8 Gy). After irradiation, mice were monitored 4–5 days/week for tumor onset. Moribund mice with difficulty in breathing were killed for necropsy. Thymi were weighed and TL was suspected when a weight >100mg was measured. TL was confirmed by histopathological examinations. The incidence of TL was plotted and analyzed by GraphPad Prism v5 software (GraphPad software, La Jolla, CA).
Histopathological analysis

Tissues were fixed in 10% buffered formalin or 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm thickness. The sections were stained with hematoxylin and eosin. In addition, tissues were frozen in OCT compound and sectioned at 4 μm thickness by cryostat. Bone marrow (BM) specimens were decalcified in a solution of 10% ethylenediaminetetraacetic acid 3-Na for 2 weeks. The primary antibodies used were as follows: anti-human CD3 mouse monoclonal Ab (F7.2.38, M2754, Dako Japan, Tokyo, Japan); anti-human terminal deoxynucleotidyl transferase (TdT) mouse monoclonal Ab (SEN28-413371, Nichirei, Tokyo, Japan); anti-mouse Ki67 antigen rat monoclonal Ab (TEC-3, M27249, Dako Japan); anti-proliferating cell nuclear antigen (PCNA) mouse monoclonal Ab (PC10, M0879, Dako Japan). For signal detection, we used Histostain-Plus Kit (Invitrogen) for CD3, TdT, PCNA or Histofine Simple Stain Mouse MAX-PO (Rat) for Ki67 staining (Nichirei).

Flow cytometry

Flow cytometry was performed using a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). Analysis of TL cells was performed by mixing TL and isolating a single cell suspension to detect expression of CD4 and CD8 cell surface antigens. Dead cells were excluded by the scatter gate. Cells were stained with APC-conjugated anti-mouse CD4 (RM4-5, 561091, BD Biosciences) antibody and fluorescein isothiocyanate-conjugated anti-mouse CD8 antibody (53–6.7, 553030, BD Biosciences) for flow cytometry.

Measurement of apoptosis

Thymocytes were isolated from mice at 4 weeks of age, with or without X-irradiation (5 Gy), and harvested by centrifugation. Nuclear DNA was extracted using a DNA Extractor TIS Kit (Wako, Osaka, Japan) and pretreated with hydroxydeoxyoxygenase (8-OHdG) assay preparation reagent set (Wako) according to the manufacturer’s instructions. The amounts of 8-OHdG were determined using a high sensitive 8-OHdG check (Japan Institute for the Control of Aging, Fukuroi, Japan) according to the manufacturer’s instructions. Data were normalized to input DNA amounts determined by UV spectrophotometer (NanoDrop ND-1000, Thermo Scientific).

Statistical analysis

Results are presented as the mean or mean ± SD. Comparisons were performed using the log-rank test and Mann–Whitney U-test. A P value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism v5 software (GraphPad software).

Results

HF and LF expression in hHF-tg mice

We recently generated hHF-tg mice that overexpressed human HF with a ubiquitous and constitutive CAG promoter (15). The hHF-tg mice carry a single copy of the transgene at chromosome 1 in their genome (S. Hasegawa et al., in preparation). We examined protein expression of HF and LF in hHF-tg mice by western blot (Figure 1). HF protein was overexpressed in the thymus, heart, muscle, kidney, liver and BM in hHF-tg mice compared with WT mice. The protein levels of LF were slightly reduced in these organs in hHF-tg mice compared with WT mice.

Accelerated radiation-induced thymic lymphoma/leukemogenesis in hHF-tg mice

To investigate whether HF overexpression accelerates radiation-induced development of TL, we irradiated 4-week-old hHF-tg and WT mice with X-rays at 1.2 Gy/week for 4 consecutive weeks (for a total dose of 4.8 Gy). All hHF-tg mice used in this study were generated by crossing hHF-tg mice with C57BL/6j mice and thus were heterozygous for the transgene. After fractionated X-irradiation, we carefully monitored the incidence of TL over a period of 200 days after the last irradiation. Unirradiated WT and hHF-tg mice were used as controls and were observed concurrently to determine the rate of spontaneous tumor development. Kaplan–Meier analysis revealed that although the two groups of unirradiated mice developed no tumors during the observation period, irradiated hHF-tg mice developed an earlier onset of TL compared with irradiated WT mice, and a significant difference in the incidence compared with WT mice (Figure 2). Median survival times of the irradiated hHF-tg were 133 days, whereas that of irradiated WT mice was 227 days. The incidence of TL in irradiated hHF-tg mice was also increased compared with that in irradiated WT mice. At 250 days after last irradiation, 80% (24/30) of irradiated hHF-tg mice developed TL compared with 53% (16/30) of irradiated WT mice.

Histopathological and immunological phenotypes of TL arising from irradiated WT and hHF-tg mice

TL that occurred in irradiated WT and hHF-tg mice was identified as a T-cell lymphoma, and confirmed by histopathological assessment (Figure 3A). TL from hHF-tg mice overexpressed HF, but not LF, compared with WT TL (Figure 3B). Infiltration of lymphoma cells into the BM was severe in hHF-tg and WT mice (Figure 3C). Iron staining suggested that there was no marked accumulation of iron in the hHF-tg TL (Supplementary Figure 1, available at Carcinogenesis Online). Flow cytometry analysis revealed that TL arising from irradiated WT and
hHF-tg mice displayed either CD4–8+ or CD4+8+ T-cell phenotypes (Figure 3D). Immunohistochemistry revealed that WT and hHF-tg lymphoma cells were strongly positive for CD3 and partially for TdT (Figure 3E). Cell proliferation of TL in irradiated hHF-tg and WT mice was evaluated by expression of Ki67 antigen, which was present in a large number of lymphoma cells from both WT and hHF-tg TLs (Figure 3F). The fraction of Ki67-positive cells in hHF-tg lymphomas was significantly higher than in WT lymphomas (Figure 3G). TLs from hHF-tg mice were strongly positive for PCNA compared with WT TLs (Figure 3H).
To investigate whether the gene expression profile in hHF-tg TLs was different from that in WT TLs, we conducted DNA microarray analyses. Comparison with normalized data between hHF-tg and WT TLs ($n = 3$ for each group) indicated that 1722 gene probes were differentially expressed, including 994 upregulated (fold changes ≥2.0) and 728 downregulated (fold change ≤−2.0) probes in hHF-tg TLs relative to WT TLs. Among them, we found significant alterations in the expression of genes that may be crucial for tumor development and progression. The representative genes are listed in Supplementary Table 1, available at Carcinogenesis Online. Analysis identified that hHF-tg TLs overexpressed molecules involved in receptor signaling (IL-4, IL-2RB, DLL1, IL-7, JAG1 and CD44), cell proliferation (cyclin D2 [CCND2], TAL1, SOX9, RUNX2, RUNX3, FGF3 and FGF9), angiogenesis (EDN1, VEGFC, SOCS3, NRARP and TGF-$\beta_2$), extracellular matrix (MMP7 and MMP15) and nucleotide metabolism (LMO1 and HOXB2). Downregulated molecules included those related to development and/or cell differentiation (TAL2, RUNX1, BMPR1B and PTK6), adhesion (NTN1, NCAM1 and NCAM2) and receptor signaling (IL-9).

Generation of 8-hydroxydeoxyguanosine in irradiated WT and hHF-tg mice

We measured the levels of 8-hydroxydeoxyguanosine (8-OHdG), a marker for oxidative DNA damage, in thymocytes after X-irradiation (Figure 4). There was no significant difference in changes of 8-OHdG levels after irradiation between WT and hHF-tg thymocytes.

Augmented apoptosis in the BM of hHF-tg mice, but not in the thymus, after fractionated irradiation

As previous studies showed that radiation-induced cell damage in BM and thymus is critical for the development of radiation-induced TLs (14), we evaluated cell damage, including apoptosis, after irradiation in these tissues from WT and hHF-tg mice. First, the $ex \ vivo$ radiation-induced apoptosis of thymocytes isolated from WT and hHF-tg mice was evaluated and showed no significant difference between the two groups (Figure 5A). Similar to the $ex \ vivo$ results, there was
Discussion

The focus of this study was to determine the role of overexpressed HF in leukemia/lymphomagenesis. We investigated whether hHF-tg mice had increased susceptibility to radiation-induced thymic lymphomagenesis compared with WT mice. The hHF-tg mice showed accelerated TL development following fractionated systemic X-irradiation compared with WT mice. This study demonstrates that HF overexpression promotes the development of radiation-induced leukemia/lymphoma.

We reported previously that HF was overexpressed in the brain of hHF-tg mice (15). HF overexpression was also found in hHF-tg embryos (S. Hasegawa et al., in preparation). In this study, we demonstrated that HF was overexpressed in various other tissues in hHF-tg mice. HF was ubiquitously overexpressed in hHF-tg mice, whereas expression of endogenous mouse LF protein was reduced in organs where the HF protein was overexpressed. This is consistent with the analyses of HF-transfected cells, where LF was downregulated by HF overexpression (16–18) and suggests that the aberrant expression of HF affects LF expression in vivo, even if the proteins originated from different species. Although HF is overexpressed systemically in hHF-tg mice, we found no apparent abnormalities in iron metabolism in hHF-tg mice. Tissue iron content was almost comparable between WT and hHF-tg mice at 4–6 weeks of age despite the overexpression of HF in the thymus and BM. No iron accumulation was observed in hHF-tg mice. Tissue iron content was almost comparable between WT and hHF-tg mice at 4–6 weeks of age despite the overexpression of HF in the thymus and BM. No iron accumulation was observed in hHF-tg mice.
increased levels of cell cycle markers such as Ki67 and PCNA and cellular proliferation was observed in hHF-tg TLs compared with WT TLs. Kiessling et al. showed that the downregulation of HF resulted in delayed tumor growth in a mouse model of T-cell lymphoma (19). These data suggest that HF overexpression might confer a proliferative advantage on lymphoma cells.

Microarray analysis revealed that genes associated with tumor development and progression, especially genes related to leukemia/lymphoma, were differentially expressed in hHF-tg TLs compared with WT TLs. For example, levels of TAL1 mRNA in hHF-tg TLs were ~2.5-fold higher than in WT TLs. TAL1, a class II basic-helix-loop-helix transcription factor, is activated by chromosomal translocation, interstitial deletion or mutation in many cases of human T-cell acute lymphoblastic leukemia (20,21). Jagged 1 (JAG1) and Delta-like 1 (DLL1), ligands in the Notch signaling pathway that are activated in human leukemia/lymphoma (22), were overexpressed in hHF-tg TLs. CCND2 mRNA was 3-fold higher in hHF-tg TLs than in WT TLs, which may explain the activated cell cycle in hHF-tg TLs. Alterations in transcriptional levels of RUNX genes were found in hHF-tg TLs. RUNX gene families encode transcription factors that are critical for hematopoietic development, and thus their aberrant expression should fail to induce normal hematopoietic cell differentiation, driving the pathway leading to a malignant state (23). Elevated expression of the LMO1 gene was observed in hHF-tg TLs. LMO1 (formerly known as RBT1N or TTG1) is a transcription unit adjacent to the breakpoints of chromosomal translocations t(11;14)(p15;q11) in T-cell leukemia (24) and thymic overexpression in mice induced T-cell acute lymphoblastic leukemia/lymphoma (25). Moreover, we found aberrant expression of cytokine signaling genes such as IL-7 and IL-2RB, which are closely related to leukemia/lymphoma development (26,27). These data clarify the genetic characteristics of hHF-tg TLs. Providing a rationale for the accelerated development of radiation-induced TL in hHF-tg mice. As hHF-tg TLs acquire common genetic alterations observed in human leukemia/lymphoma, this model may provide insights into the role of HF overexpression in the development of radiation-dependent and radiation-independent human leukemia/lymphoma. Recently, Bueno et al. reported gene expression profiles in γ-irradiation-induced mouse T-cell lymphomas (28). Interestingly, they showed that HF expression was deregulated in the lymphomas. This further supports our findings that aberrant HF expression was closely linked to radiation-induced leukemia/lymphomagenesis. In addition, we found that transcripts of hepcidin antimicrobial peptide, an iron-related molecule, were upregulated in hHF-tg TLs. HF expression and X-irradiation could affect mitochondrial function (32–34). To access the mitochondrial dysfunction in hHF-tg mice, we evaluated the production of reactive oxygen species through the levels of 8-OHdG after irradiation. No significant differences in 8-OHdG production were observed after irradiation between the two groups of mice, suggesting that acceleration of radiation-induced TL development in hHF-tg mice is not attributed to differential mitochondrial function.

Augmented apoptosis in hHF-tg BM was observed after irradiation. These results suggest that BM from hHF-tg mice is more sensitive to radiation-induced apoptosis than that from WT mice and suggests that more severe cell damage or augmented apoptosis in BM promotes radiation-induced TL. Of note, two recent investigations reported that apoptosis in BM cells could drive γ-irradiation-induced thymic lymphomagenesis (35,36), a result that is consistent with the data presented here and that supports the notion that augmented apoptosis of BM cells can accelerate the radiation-induced development of TL. Analysis of the iron content of WT and hHF-tg BM by inductively coupled plasma mass spectrometry indicated there was no significant difference in iron content between WT and hHF-tg BM cells. Therefore, it is unlikely that the accumulation of iron was solely responsible for the enhanced radiation-induced apoptosis. Rather, we favor the possibility that HF overexpression itself may contribute to the augmented apoptosis observed in hHF-tg mice. Interestingly, Della Porta et al. reported higher levels of HF in patients with myelodysplastic syndrome (13), a preleukemic disorder characterized by progression to acute myeloid leukemia, displaying exaggerated apoptosis and inefficient hematopoiesis in its early stages (37). Thus, HF overexpression may accelerate progression from myelodysplastic syndrome to a fully malignant state.

Although we showed augmented radiation-induced apoptosis in hHF-tg mice, the role of HF in apoptosis remains controversial. Bresgen et al. recently suggested that the secretory form of HF has proapoptotic activity (38,39). However, other groups (10,40,41) have also reported that cytosolic HF serves as an antiapoptotic protein by suppressing iron-mediated oxidative stress. Therefore, the role of HF in apoptosis may depend on cell type, form or intracellular localization.

In summary, we provide in vivo evidence that HF overexpression accelerated radiation-induced development of TL. On the basis of the present results, we propose that overexpressed HF may increase the risk of radiation-induced leukemia/lymphoma. These findings may have important implications for the prevention of malignant hematologic diseases in humans.

Supplementary material

Supplementary Table 1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/

Funding

This work was supported in part by President’s grant of National Institute of Radiological Sciences to S.H.

Acknowledgements

We thank Ramila Paudel, Hitomi Sudo for their expert technical support and Toshiaki Kokubo for helpful comments regarding the histopathological studies.

Conflict of Interest Statement: None declared.

References


Received March 10, 2012; revised July 21, 2012; accepted July 24, 2012