Leukemic potential of doubly mutant Nf1 and Wv hematopoietic cells

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The development of molecularly targeted treatments of adult leukemias warrants investigation of these targets in similar pediatric leukemias. The NF1 tumor suppressor gene, which encodes a GTPase activating protein for p21ras, is frequently inactivated in juvenile myelomonocytic leukemia (JMML). Other patients with JMML acquire activating RAS gene mutations. Recipient mice reconstituted with Nf1<sup>−/−</sup> fetal hematopoietic cells develop a myeloproliferative disease (MPD) that models the human disease. JMML arises from clonal expansion of a hematopoietic stem cell, and JMML cells and murine Nf1<sup>−/−</sup> hematopoietic cells are hypersensitive to granulocyte macrophage-colony stimulating factor and KitL, the ligand for c-kit. We generated embryos doubly mutant for the W<sup>v</sup> allele of c-kit and NF1 to ask if reduction of c-kit activity would delay or prevent the development of MPD. Despite a reduction in c-kit activity to approximately 10% of wild-type levels, Nf1<sup>−/−</sup>; W<sup>v</sup>/W<sup>v</sup> cells induced MPD in recipient mice. (Blood. 2003;101:1984-1986)

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Introduction

Mutations in the NF1 tumor suppressor gene cause neurofibromatosis type I (NF1). NF1 encodes neurofibromin, which negatively regulates p21<sub>RAS</sub> activity by accelerating the conversion of active p21<sub>RAS</sub>-GTP to inactive p21<sub>RAS</sub>-GDP. Children with NF1 are at markedly increased risk of developing juvenile myelomonocytic leukemia (JMML), for which existing treatments are largely ineffective. Although NF1 knockout mice (Nf1<sup>−/−</sup>) die in utero around day embryonic (E)13.5, recipients of transplanted Nf1<sup>−/−</sup> fetal hematopoietic stem cells (HSCs) develop myeloproliferative disease (MPD) that closely models JMML. This model has been exploited for preclinical studies of therapeutics that target hyperactive p21<sub>RAS</sub>. Murine Nf1<sup>−/−</sup> fetal liver cells form extensive numbers of myeloid progenitors in methyccelulose cultures containing low concentrations of kit ligand (KitL), and we recently found that Nf1<sup>−/−</sup> HSCs have a self-renewal advantage in vivo. KitL is a potent survival and proliferative growth factor for both HSCs and myeloid progenitors (reviewed in Broudy). Inasmuch as JMML often arises from clonal expansion of an HSC and that Nf1<sup>−/−</sup> myeloid progenitors are hypersensitive to KitL, we hypothesized that genetic inhibition of the KitL/c-kit pathway would alter the progression of MPD in recipients of Nf1<sup>−/−</sup> fetal liver cells. Evaluating c-kit as a therapeutic target is an important priority as the tyrosine kinase inhibitor (imatinib mesylate) induces regression in gastrointestinal stromal tumors (GISTs), a malignancy characterized by mutations that constitutively activate the c-kit kinase. To assess if impairing c-kit function would modulate the MPD induced by NF1 inactivation, we generated fetal stem cells that were doubly mutant at NF1, and the dominant white spotting (W) locus, which encodes c-kit, performed adoptive transfers and monitored the recipient mice for development of MPD. We find that the W<sup>v</sup> mutation, which reduces c-kit kinase activity by approximately 90%, does not suppress the capacity of Nf1<sup>−/−</sup> fetal liver cells to induce MPD.

Study design

Animals and adoptive transfer procedure

Nf1<sup>−/−</sup> mice in a C57BL/6.129 background were backcrossed for 13 generations into C57BL/6d mice, which were purchased from Jackson Laboratories (Bar Harbor, ME). W/W<sup>v</sup> mice were purchased from Jackson Laboratories in a C57BL/6d strain. Studies were conducted with a protocol approved by the Indiana University Animal Care and Use Committee. The NF1 and W<sup>v</sup> alleles were genotyped as previously described. The crosses used to generate day-E13.5 fetal livers, which contain the experimental groups, are outlined here. Filial (F0): Nf1<sup>−/−</sup>; W<sup>v−/−</sup> × Nf1<sup>−/−</sup>; W<sup>v−/−</sup>. F1: Nf1<sup>−/−</sup>; W<sup>v−</sup> × Nf1<sup>−/−</sup>; W<sup>v−</sup>. F2 experimental groups: Nf1<sup>−/−</sup>; W<sup>v−/−</sup>, Nf1<sup>−/−</sup>; W<sup>v−</sup>, Nf1<sup>−/−</sup>; W<sup>v−/−</sup>, W<sup>v−/−</sup>, W<sup>v−</sup>.

Syngeneic recipients were given transplants of 3 million day-E13.5 fetal liver cells generated from the intercrosses following irradiation with 1100 cGy as previously described. Animals that underwent transplantation were followed for signs of MPD, and photomicrographs of spleen sections were taken with an Olympus DP11 (Melville, NY).

Progenitor assays

Colonies derived from myeloid progenitors were enumerated exactly as described. Recombinant granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3), and KitL were purchased from...
Peprotech (Rocky Hill, NJ), and recombinant IL-1 and M-CSF were obtained from R&D Systems (Minneapolis, MN). Cells and recombinant growth factors were added to agar for growth of low proliferating potential–colony forming cells (LPP-CFCs) and high proliferating potential–colony forming cells (HPP-CFCs), and the solution was thoroughly admixed before plating. LPP-CFCs and HPP-CFCs were enumerated in 8% CO2 and 5% O2. LPP-CFCs and HPP-CFCs were scored by indirect microscopy on days 7 and 14, respectively.

### Results and discussion

Of 300 embryos generated from the $W^v\times Nf1$ intercross, only 3 were doubly mutant (W$^{v-/-};$W$^v$) at day E13.5, and fetal liver cell numbers were markedly reduced. This is consistent with previous studies using $W^v/W^v$ mice.12 Despite these obstacles, one recipient received a transplant successfully from each doubly mutant embryo; 2 of the recipients developed white blood cell (WBC) counts of 0.045 and 0.050 $\times$ $10^9/L$ ([45 000 and 50 000 per mm$^3$] compared with an average WBC count of 0.017 $\times$ $10^9/L$ (17 000 per mm$^3$) in recipients of wild-type fetal liver cells; n = 10). Each mouse died at 2 months of age and had gross splenomegaly upon inspection, which is consistent with MPD previously observed in recipients of $Nf1^{v-/-}$-deficient fetal liver cells.6,9 The third recipient of transplanted $Nf1^{v-/-};W^v/W^v$ cells survived to 4 months of age, which allowed a more detailed analysis. The spleen weight and cellularity were markedly increased compared with the wild type and $W^v/W^v$ controls and were comparable to mice that received transplants of $Nf1^{v-/-}$ fetal stem cells (Table 1). The MPD in mice that received transplants of $Nf1^{v-/-}$ cells is characterized by a dramatic increase in the numbers of splenic HPP-CFCs and LPP-CFCs.9 The numbers of HPP-CFCs and LPP-CFCs were similar in the spleens of recipients injected

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with singly mutant Nf1−/− or Nf1−/−;W/W mice and were increased 30-fold (HPP-CFCs) and 10-fold (LPP-CFCs) versus mice that received transplants of wild-type or W/W cells (Table 1). Finally, the spleens of the recipients of transplanted Nf1−/−;W/W or Nf1−/− cells demonstrated myeloid cell infiltration and effacement of splenic architecture, which is consistent with development of MPD (Figure 1A-D).

Studies in which bone marrow cells from primary recipients were transferred into secondary hosts showed that Nf1 inactivation is associated with enhanced proliferative potential in repopulating stem cells. To examine whether the reduction in c-kit activity altered this previously defined gain of function potential, we isolated low-density marrow mononuclear cells from each mouse of the 4 F2 experimental groups and transplanted these cells into secondary recipients. Mice were killed at 6 months of age, and spleens were examined for evidence of MPD. All recipients of transplanted Nf1−/−;W/W or Nf1−/− cells (n = 8) developed a dramatic myeloid cell infiltration of the spleen consistent with MPD as previously observed in the primary recipients and in prior studies. A representative spleen section from each experimental group is shown in Figure 1E-H.

In vitro hypersensitivity to GM-CSF in methylcellulose cultures is a hallmark of human JMML blood and marrow cells, a characteristic that is also observed in Nf1−/− fetal liver cells. Experiments in which Nf1 and Gmcsf mutant mice were mated to generate cells for adoptive transfer demonstrated a central role of this aberrant response to GM-CSF in the pathogenesis of MPD. Nf1−/− hematopoietic cells are also hypersensitive to KitL in colony-forming assays, and KitL and GM-CSF act synergistically to enhance myeloid progenitor growth from wild-type and Nf1−/− cells. These data, the availability of imatinib mesylate for clinical trials, and the poor prognosis for patients with JMML led us to ask if reducing c-kit activity might modulate the course of MPD in recipients of Nf1−/− fetal liver cells. We pursued a genetic approach to this question because imatinib inhibits c-Abl and β chain of the platelet-derived growth factor receptor in addition to c-kit. Our data showing that Nf1−/−;W/W fetal liver cells induced MPD suggest that this disorder is not dependent on c-kit signaling. Alternatively, 10% of normal c-kit activity may be sufficient for leukemogenesis, whereas complete inhibition might interfere with the growth of JMML cells in vivo. However, this possibility is highly unlikely since W/W;Nf1−/− hematopoietic cells (W/W mutants have a 70% reduction in c-kit activity vs 90% for W strains) are not hypersensitive to KitL in vivo as determined in both myeloid colony-forming assays and competitive repopulation assays, which measure HSC function (data not shown). It also possible that c-kit inhibitors will be ineffective as single agents in JMML, but will show synergistic activity with drugs that target GM-CSF signaling such as the peptidomimetic E12R. These hypotheses can be tested by treating mice that received transplants of Nf1−/− cells with imatinib mesylate or with other pharmacologic inhibitors of c-kit. Recently, a heterozygous environment was shown to contribute to the development of neurofibromas in mice in which Nf1 was selectively ablated in Schwann cells. Heterozygous Nf1 mutant cells infiltrate these lesions, and these cells demonstrate deregulated p21 signaling in response to KitL.

Thus, although our data do not support a role for c-kit inhibitors in JMML, drugs that interfere with this pathway might prove useful for treating other complications of Nf1.

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References