Brief Definitive Report

Genetic and Biochemical Evidence that Haploinsufficiency of the Nf1 Tumor Suppressor Gene Modulates Melanocyte and Mast Cell Fates In Vivo

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Abstract

Neurofibromatosis type 1 (NF1) is a common autosomal-dominant disorder characterized by cutaneous neurofibromas infiltrated with large numbers of mast cells, melanocyte hyperplasia, and a predisposition to develop malignant neoplasms. NF1 encodes a GTPase activating protein (GAP) for Ras. Consistent with Knudson's "two hit" model of tumor suppressor genes, leukemias and malignant solid tumors in NF1 patients frequently demonstrate somatic loss of the normal Nf1 allele. However, the phenotypic and biochemical consequences of heterozygous inactivation of Nf1 are largely unknown. Recently neurofibromin, the protein encoded by NF1, was shown to negatively regulate Ras activity in Nf1−/− murine myeloid hematopoietic cells in vitro through the c-kit receptor tyrosine kinase (dominant white spotting, W).

Since the W and Nf1 locus appear to function along a common developmental pathway, we generated mice with mutations at both loci to examine potential interactions in vivo. Here, we show that haploinsufficiency at Nf1 perturbs cell fates in mast cells in vivo, and partially rescues coat color and mast cell defects in W41 mice. Haploinsufficiency at Nf1 also increased mast cell proliferation, survival, and colony formation in response to Steel factor, the ligand for c-kit. Furthermore, haploinsufficiency was associated with enhanced Ras–mitogen-activated protein kinase activity, a major downstream effector of Ras, via wild-type and mutant (W41) c-kit receptors. These observations identify a novel interaction between c-kit and neurofibromin in vivo, and offer experimental evidence that haploinsufficiency of Nf1 alters both cellular and biochemical phenotypes in two cell lineages that are affected in individuals with NF1. Collectively, these data support the emerging concept that heterozygous inactivation of tumor suppressor genes may have profound biological effects in multiple cell types.

Key words: c-kit • mast cell • heterozygous • neurofibromatosis • tumor suppressor

Introduction

Neurofibromin, the protein encoded by neurofibromatosis type 1 (NF1), negatively regulates Ras as output by accelerating the conversion of Ras-GTP to Ras-GDP (1, 2). The murine c-kit receptor (3) and its ligand, Steel factor (4, 5), are components of a signaling pathway that is essential for murine hematopoiesis, melanogenesis, and gametogenesis.

These proteins are encoded by the dominant white spotting (W) and Steel (Sl) loci, respectively, and ligand binding to c-kit activates Ras in myeloid lineage cells (for a review, see reference 6). Children with NF1 are at a markedly increased risk of developing juvenile myelomonocytic leukemia (JMML [7]). Genetic and biochemical analyses of these leukemias strongly support the hypothesis that Nf1 functions as a tumor suppressor gene in immature myeloid cells by negatively regulating Ras as output. Similarly, ~10% of heterozygous Nf1−/− mice spontaneously develop a JMML-like myeloproliferative disorder (MPD) during the

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second year of life with loss of the wild-type Nf1 allele (8). In contrast to patients with Nf1, Nf1+/− mice neither manifest pigmentary abnormalities nor develop neurofibromas (8). Although homozygous Nf1 knockout mice (Nf1−/−) die in utero around embryonic day (E)13.5 from complex cardiovascular defects (8, 9), adoptive transfer of E13.5 Nf1−/− fetal liver hematopoietic stem cells into irradiated syngeneic recipients consistently induces the JM M L-M PD (10). Interestingly, we have shown recently that murine Nf1−/− fetal liver cells form excessive numbers of myeloid progenitor colonies in methylcellulose cultures containing low concentrations of Steel factor. Mitogen-activated protein kinase, a downstream target of Ras-GTP, is activated at both cell, and melanocyte lineages in some of the pathological conditions at both cell, and melanocyte lineages in some of the pathological conditions. In unstimulated (MAP) kinase, a downstream target of Ras-GTP, is activated at both cell, and melanocyte lineages in some of the pathological conditions.

Materials and Methods

Animals. Nf1+/− mice were obtained from Dr. Tyler Jacks at the Massachusetts Institute of Technology (Cambridge, MA) in a C57BL/6.129 background, and were backcrossed for 13 generations into the C57BL/6 strain. C57BL/6+/−,+;W41/+ mice were obtained from the Jackson Laboratory. These studies were conducted with a protocol approved by the Indiana University Laboratory Animal Research Center. The Nf1 allele was genotyped as described previously (11, 12). The W41 genotyping was inferred from the characteristic mottled, white coat color in W41/− mice, and a white abdominal spot on W41/+/ mice. Mice and W41 genotypes used in these experiments are outlined below:

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>F0: Nf1+/−;+</td>
<td>Nf1+/− mice used to generate F2 progeny used in these experiments</td>
</tr>
<tr>
<td>F1: Nf1+/−;−</td>
<td>Nf1+/−, W41/+/+, +/+;W41/+ mice</td>
</tr>
<tr>
<td>F2: Nf1+/−;−;W41/+</td>
<td>W41/+ mice</td>
</tr>
</tbody>
</table>

A analysis of cutaneous mast cells 1-cm sections of ears and dorsal skin were removed, fixed in buffered formalin, and processed in paraffin-embedded sections. Specimens were stained with hematoxylin-eosin to assess routine histology, and with Giemsa to identify mast cells. Some specimens were stained with Fontana-Mason to differentiate melanin-containing cells from mast cells. Cutaneous mast cells (Giemsa-positive, Fontana-Mason-negative) were quantitated in a blinded fashion by counting the distal 5 mm of ears or dorsal skin.

Peritoneal lavages and mast cell colony assays. Peritoneal cells from 8-wk-old mice were collected as described previously (13). 10-ml peritoneal lavages were then centrifuged and stained with toluidine blue to quantify total numbers of mast cells per 10 ml lavage. To examine the proliferation of peritoneal mast cell progenitors, mast cell colony assays were done in triplicate. In brief, 1 ml of culture mix containing 1 × 10⁴ peritoneal cells, α-MEM, 1.2% methylcellulose (Terry Fox Laboratory), 30% fetal bovine serum (HyClone Laboratories), 1% deionized fraction V BSA (Sigma Chemical Co.), 10⁻⁴ M mercaptoethanol (Sigma Chemical Co.), 10 ng/ml recombinant murine IL-3, and 100 ng/ml recombinant murine Steel factor (PeproTech) was plated in each 35-mm suspension culture dish. Colonies were determined on day 14 of incubation by in situ observation using an inverted microscope. To assess the accuracy of in situ identification of the colonies, individual colonies were taken with an Eppendorf micropipette under direct microscope visualization, spread on glass slides using cytocentrifuge, and stained with May-Grünwald-Giemsa stain and Alcian blue/safranin stain for mast cells.

Bone marrow mast cell culture and survival assay. Six lines from each of the four genotypes were generated and used for cell survival and proliferation assays. Bone marrow mast cells (BM M Cs) were cultured as described, with minor modifications (14), and homogeneity of BM M Cs was determined by Giemsa staining. Aliquots of cells were also stained with Alcian blue and safranin to confirm that they were mast cells. Furthermore, FACScan analysis revealed similar forward and side light scatter characteristics and the same percentage of c-kit+ cells in BM M Cs of all four genotypes (data not shown). The mast cell survival assay was done as follows: BM M Cs from each genotype were deprived of growth factors for 24 h, and 3 × 10⁵ cells were plated in 24-well dishes in serum-free RPMI containing 1% glucose and 100 ng/ml of recombinant murine Steel factor in a total volume of 1 ml. The number of surviving cells was determined by trypan blue exclusion at 48 h of culture in a 37°C, 5% CO₂, humidified incubator.

Results

Haploinsufficiency at Nf1. Partially R stores the C oat C olor Deficiency in W41 Mice. W mice display varying degrees of sterility, mast cell hypoplasia, anemia, and coat color deficiency correlating with the residual kinase activity of the mutant receptor (16, 17). Mice homozygous for a point mutation in the
cytoplasmic domain of the c-kit receptor (W41) have partial inactivation of the c-kit receptor tyrosine kinase, resulting in an abnormal mottled, white coat color (17). We crossed W41 and Nfl mice and found that animals heterozygous at Nfl and homozygous for the W41 mutation (Nfl+/−; W41/W41) displayed a 60–70% restoration of coat color (Fig. 1 A). This finding was consistent in >150 F2 progeny carrying mutations at both loci. Thus, haploinsufficiency at Nfl partially corrects the aberrant pigmentation pattern of W41/W41 mice.

Haploinsufficiency at Nfl Increases Peritoneal and Cutaneous Mast Cell Numbers in Wild-Type and W41 Mice. Peritoneal mast cell numbers in Nfl mice showed a twofold increase in numbers of cutaneous mast cells compared with +/+;W41/W41 mice (Table I). Nfl+/−;+/+ mice also had a modest, though statistically insignificant, increase in cutaneous mast cell numbers compared with wild-type mice (Table I). Giemsa-stained ear biopsies from mice of the four W and Nfl genotypes are shown in Fig. 1 C. Ear biopsies from Nfl+/−;W41/W41 mice showed a twofold increase in numbers of cutaneous mast cells compared with +/+;W41/W41 mice (Table I).

To investigate whether heterozygous inactivation of Nfl could modulate the deficiency of this lineage, we compared numbers of peritoneal mast cells harvested from Nfl+/−; W41/W41 animals to those taken from singly mutant mice (+/+;W41/W41). We also compared peritoneal mast cell numbers in Nfl+/−;+/+ and wild-type mice. Cells isolated by peritoneal lavage were stained with toluidine blue to identify mast cells. Representative cytospins from individual mice from each of the four Nfl and W genotypes are shown in Fig. 1 B. Peritoneal mast cell numbers in Nfl+/−; W41/W41 mice were 40-fold higher than in +/+; W41/W41 littermates (Table I). Importantly, in addition to restoring peritoneal mast cell numbers to ~10% of wild-type levels in W41/W41 mice, heterozygous inactivation of Nfl significantly increased mast cell numbers in animals that were wild-type at the W locus (Table I). We performed similar experiments to determine if haploinsufficiency at Nfl increased numbers of cutaneous mast cells in the mutant W41/W41 and wild-type backgrounds. Giemsa-stained ear biopsies from mice of the four W and Nfl genotypes are shown in Fig. 1 C. Ear biopsies from Nfl+/−;W41/W41 mice showed a twofold increase in numbers of cutaneous mast cells compared with +/+;W41/W41 mice (Table I). Giemsa-stained biopsies obtained from a second site (dorsal skin) revealed similar differences in cutaneous mast cell numbers between the genotypes (data not shown). The increased numbers of peritoneal and cutaneous mast cells in Nfl+/− mice provide additional evidence that haploinsufficiency augments signaling through the c-kit receptor in vivo.

Haploinsufficiency at Nfl Increases Colony Formation, Proliferation, and Survival of Mast Cells in Response to Steel Factor in W41 and Wild-Type Mice. IL-3 and Steel factor support growth of mast cells, and we have shown that Steel factor is present in the peritoneal cavity, though at lower concentration than IL-3 (3). Colony formation and proliferation assays were performed to determine if Steel factor supports mast cell growth in the absence of IL-3. The number of peritoneal colony forming units (CFUs) per mouse was increased in Nfl+/− mice compared with +/+ and Nfl−/− mice (Fig. 2 A). Colony formation increased 15- to 30-fold in Nfl+/−; W41/W41 mice compared with +/+;W41/W41 mice (Fig. 2 B). These findings are consistent with previous reports that Steel factor is an important growth factor for mast cells in vivo and in vitro (3, 18). Colony formation was also increased in W41/W41 mice compared with +/+ W41/W41 littermates. The addition of Steel factor to the colony formation assay did not increase colony formation in +/+;W41/W41 or Nfl+/−;W41/W41 mice. This result suggests that Steel factor is not able to rescue the loss of Tyk2 or NF1 function in these mice.

Figure 1. Effect of haploinsufficiency of Nfl on coat color and total numbers of cutaneous and peritoneal mast cells. (A) Coat color pattern of a representative mouse from each of the following genotypes: +/+; +/+; Nfl+/−; +/+; +/+; Nfl+/−; +/+; +/+; W41/W41, and Nfl+/−; W41/W41. Haploinsufficiency at Nfl partially corrects the coat color deficiency in mice homozygous for the W41 allele in a C57BL/6 genetic background. (B) Representative cytospins from peritoneal lavages stained for mast cells from individual mice of the four Nfl and W genotypes. Peritoneal cells were stained with toluidine blue to quantify the total number of mast cells per peritoneal lavage. A higher magnification of a representative mast cell is shown in the inset of the wild-type mouse (original magnification: ×200). Bar (inset) 10 µm. Bar (far right) 30 µm. (C) Representative ear biopsies stained for cutaneous mast cells from individual mice of the four Nfl and W genotypes. Specimens were stained with hematoxylin-eosin to assess routine histology, and with Giemsa to identify mast cells. Ear biopsies were stained with Fontana-Masson to differentiate melanin-containing cells from mast cells. Cutaneous mast cells (Giemsa-positive, Fontana-Masson-negative) were quantitated in a blinded fashion by counting the distal 5 mm of ears. Black arrows indicate Giemsa-positive mast cells, and open arrows indicate Fontana-Masson melanin-containing cells. Bar, 35 µm.
Table I. Effects of W and Nf1 Genotypes on Mast Cell Numbers and Mast Cell Colony Growth

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of peritoneal mast cells (×10⁶)</th>
<th>No. of cutaneous mast cells (mm²)</th>
<th>No. of mast cell colonies (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>780 ± 10</td>
<td>25.6 ± 7</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>Nf1/+−</td>
<td>1,000 ± 14*</td>
<td>31 ± 2</td>
<td>117 ± 9*</td>
</tr>
<tr>
<td>+/+</td>
<td>2 ± 1</td>
<td>6 ± 1</td>
<td>0</td>
</tr>
<tr>
<td>W41/W41</td>
<td>81 ± 17*</td>
<td>14 ± 2*</td>
<td>8 ± 0.8*</td>
</tr>
</tbody>
</table>

*P < 0.05 for comparison of Nf1+/− and Nf1+/+ in both W41/W41 and wild-type genetic backgrounds by Student’s paired t test.

N os of peritoneal and cutaneous mast cells were quantitated from peritoneal lavages and ear biopsies. Results represent mean numbers of mast cells ± SEM from six animals in each genotype. Peritoneal cells were cultured for the growth of mast cell colonies as described (reference 13), and results represent the mean number of mast cell colonies ± SEM of six independent experiments.

Figure 2. Effect of haploinsufficiency of Nf1 and W on the survival and proliferation of BM M C Cs in response to Steel factor. (A) Proliferation of BM M C Cs from mice of the four Nf1 and W genotypes in response to recombinant murine Steel factor. After deprivation of growth factors for 24 h, 2 × 10⁶ cells/ml were plated in triplicate in 24-well dishes in RPMI containing 1% glutamine, 10% fetal bovine serum, and 100 ng/ml of Steel factor in a total volume of 1 ml as described previously (reference 29). After 1 and 3 d, viable cells were counted using a hemocytometer and expressed as a percentage of input cells. *P < 0.05, Nf1+/−; W41/W41 vs. +/+; W41/W41 and Nf1+/−; +/+ vs. +/+; +/+ cells by Student’s paired t test. (B) Percent survival of BM M C Cs of the four Nf1 and W genotypes. After deprivation of growth factors for 24 h, 3 × 10⁵ cells of each genotype were plated in RPMI containing 1% BSA and 100 ng/ml of recombinant murine Steel factor. The number of surviving cells was determined by trypan blue exclusion and expressed as a percentage of input cells. *P < 0.05, Nf1+/−; W41/W41 vs. +/+; W41/W41 and Nf1+/−; +/+ vs. +/+; +/+ cells by Student’s paired t test.
type and W41/W41 BMMCs in liquid cultures containing 10% FCS and exogenous Steel factor.

Steel factor acts as both a mitogen and a survival factor for mast cells (5, 17, 19). Mast cells derived from different lines of W mutant mice display reduced or no survival when cultured in the presence of Steel factor alone, which correlates with residual receptor tyrosine kinase activity (17, 19).

To test this hypothesis, we stimulated primary BMMCs with Steel factor, and measured p42 MAP kinase. Lineages. To test this hypothesis, we stimulated primary BMMCs with Steel factor alone, which correlated with c-kit–induced Ras output by reducing neurofibromin expression of both W genotypes demonstrated increased survival compared with Nf1+/− cells after 48 h of culture in serum-free media in response to exogenous Steel factor. Since this assay has been shown to correlate with c-kit receptor tyrosine kinase activity (19), we quantitated surface expression of c-kit on the four W and Nf1 genotypes by fluorescence cytometry to ensure that these differences in cell survival were not explained by variable levels of receptor expression. No differences were observed (data not shown).

Haploinsufficiency at Nf1 increases MAP kinase activity in response to Steel factor in Wild-T-type and W41 mice. The data presented above provide evidence that haploinsufficiency of Nf1 augments the proliferation and survival of wild-type and W41/W41 mast cells in vitro and in vivo. Partial restoration of normal coat color in Nf1+/−; W41/W41 mice indicates that this effect is not restricted to hematopoietic cells, but also includes melanocytes. The Ras–MAP kinase pathway is an important downstream target of c-kit receptor activation, and neurofibromin negatively regulates Ras as signaling by functioning as a GTPase activating protein (GAP) for Ras (20, 21). The phenotypic data presented above suggest a model whereby heterozygous inactivation of Nf1 enhances c-kit–induced Ras as output by reducing neurofibromin levels (and GAP activity for Ras) in susceptible cell lineages. To test this hypothesis, we stimulated primary BMMCs with Steel factor, and measured p42 MAP kinase. MAP kinase protein levels were similar in BMMCs of all genotype combinations (data not shown). Nf1+/−; W41/W41 BMMCs demonstrated a fivefold greater increase in MAP kinase activity from baseline 5 min after the addition of Steel factor relative to +/+; W41/W41 BMMCs (Fig. 3). Indeed, haploinsufficiency of Nf1 restored the ability of the mutant W41 c-kit receptor to activate MAP kinase to wild-type levels at the 5-min time point (Fig. 3). Similarly, Nf1+−/+ BMMCs had a twofold greater increase in MAP kinase activity from baseline compared with wild-type mast cells that was sustained at both tested time points (Fig. 3). These biochemical data indicate that the phenotypic effects in Nf1+/− mast cells correlate with enhanced signaling through a major downstream effector of Ras–GTP.

**Discussion**

In these experiments, we present genetic, cellular, and biochemical data demonstrating that neurofibromin negatively regulates signaling through the c-kit receptor tyrosine kinase in a haploinsufficient state. Dermal cafe au lait macules, learning disabilities, and the development of multiple cutaneous neurofibromas are major nonmalignant pathologic complications of NF1. Although the finding of constitutional heterozygosity (LOH) in some neurofibromas supports the “two hit” tumor suppressor model (22, 23), many of these lesions retain the normal NF1 allele. Other features of neurofibroma biology, including the very large numbers of lesions found in some patients, their self-limited growth, and the low propensity of these tumors to undergo malignant degeneration are also consistent with a possible dosage effect on cell growth. Neurofibromas are infiltrated with mast cells that have been hypothesized to promote growth by releasing mediators that act locally upon Schwann cells, endothelial cells, and fibroblasts (24). Treatment with mast cell stabilizers is associated with a reduction in pruritus in some patients with NF1, and represents the only known medical treatment that alters the growth of neurofibromas (24). Given the importance of Steel factor in regulating multiple mast cell functions, our data showing that haploinsufficiency at Nf1 alters mast cell numbers, survival, and Ras signaling in wild-type and W41/W41 mice in response to Steel factor implicate deregulated mast cell function as potentially important in neurofibroma formation.

In a recent study, Hemesath et al. (25) implicated the Microphthalmia (Mi) transcription factor as a direct target of MAP kinase activation in response to c-kit. The observation that haploinsufficiency at Nf1 effects a 60–70% rescue of the pigmentary defect seen in W41/W41 mice further supports the central role of this pathway in melanocyte development, and is consistent with the marked enhancement of
Steel factor–induced MAP kinase activation that we observed in Nf1+/− mast cells. Although the molecular basis of cutaneous melanocyte hyperplasia in Nf1 is poorly understood, our data suggest that melanocytes from Nf1+/− mice could offer an attractive system for discerning the role of Nf1 in examining the c-kit-MAP kinase-M1 pathway.

The relative contributions of homozygous Nf1 inactivation and of haploinsufficiency to the pathologic complications of Nf1 remain incompletely understood. Genetic analysis of malignant peripheral nerve sheath tumors (MPNSTs), pheochromocytomas, and myeloid leukemias from individuals with Nf1 and Nf1+/− mice have demonstrated frequent somatic loss of the normal allele (8, 26–29). Homozygous inactivation of Nf1 in a human MPNST, in several leukemias, and in a variety of murine tumors provides formal proof that the gene functions as a tumor suppressor in a subset of cancers (8, 30). Although these data confirm that tumorigenesis follows the Knudson paradigm (31) in some patients and in Nf1 mice, many tumors do not show loss of LOH, and it is uncertain if inactivation of both alleles is a prerequisite for tumor formation. In a recent study of heterozygous p53 knockout mice, some tumors retained a functional p53 allele (32). Tumor formation in p27 knockout mice is also associated with haploinsufficiency (33). The phenotypes that we have detected in Nf1+/− cells of two lineages suggest that haploinsufficiency may confer a growth advantage that could contribute to tumorigenesis by pathways that do not require inactivation of the normal allele. Full genetic and biochemical characterization of tumors from patients with Nf1 and from Nf1+/− mice that retain heterozygosity is required to address this possibility. Finally, the finding that haploinsufficiency at Nf1 has dramatic phenotypic consequences in two cell lineages that are affected in Nf1 patients has important therapeutic implications. In particular, if diseased cells retain a functional Nf1 allele, increasing neurofibromin–specific GAP activity is an attractive strategy for preventing or treating the complications of Nf1.

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