Rapid Communication

Myeloid Malignancies Induced by Alkylating Agents in Nf1 Mice

By Nidal Mahgoub, Brigit R. Taylor, Michelle M. Le Beau, Mary Gratiot, Katrin M. Carlson, Susan K. Atwater, Tyler Jacks, and Kevin M. Shannon

Therapy-related acute myeloid leukemia and myelodysplastic syndrome (AML and MDS) are severe late complications of treatment with genotoxic chemotherapeutic agents. Children with neurofibromatosis type 1 (NF1) are predisposed to malignant myeloid disorders that are associated with inactivation of the Nf1 tumor suppressor gene in the leukemic clone. Recent clinical data suggest that Nf1 might be associated with an increased risk of t-AML after treatment with alkylating agents. To test this hypothesis, we administered cyclophosphamide or etoposide to cohorts of wild-type and heterozygous Nf1 knockout mice. Cyclophosphamide exposure cooperated strongly with heterozygous inactivation of Nf1 in myeloid leukemogenesis, while etoposide did not. Somatic loss of the normal Nf1 allele correlated with clinical disease and was more common in 129/Sv mice than in 129/Sv × C57BL/6 animals. Leukemic cells showing loss of heterozygosity at Nf1 retained a structural allele on each chromosome 11 homolog. These studies establish a novel in vivo model of alkylator-induced myeloid malignancy that will facilitate mechanistic and translational studies.

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MATERIALS AND METHODS

Animal care. Mice were housed in the University of California, San Francisco (UCSF) Animal Care facility and were examined regularly by one of the investigators. Cyclophosphamide and etoposide were prepared by the UCSF pharmacy and were administered by one of the investigators. Cyclophosphamide exposure cooperated strongly with heterozygous inactivation of Nf1 in myeloid leukemogenesis, while etoposide did not. Somatic loss of the normal Nf1 allele correlated with clinical disease and was more common in 129/Sv mice than in 129/Sv × C57BL/6 animals. Leukemic cells showing loss of heterozygosity at Nf1 retained a structural allele on each chromosome 11 homolog. These studies establish a novel in vivo model of alkylator-induced myeloid malignancy that will facilitate mechanistic and translational studies.

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3617
investigators. Mice were weighed at the beginning of the study and weekly thereafter to adjust the drug doses. Complete blood counts (CBCs) were performed on blood samples collected from tail veins in an automated cell counter. The accuracy of abnormal blood counts was verified by direct examination of stained smears. The study procedures were reviewed and approved by the UCSF Committee for Animal Research.

Treatment and monitoring. We mated Nf1+/− and wild-type (Nf1+/+) mice and genotyped the offspring by Southern blot analysis of tail DNA. The initial group of mice were from the inbred 129/Sv strain in which the Nf1 mutation was created.16 To perform LOH analysis at loci other than Nf1 in alkylator-treated mice, F1 offspring of a cross between the 129/Sv and C57BL/6 strains were used in the latter part of the study. Nf1+/− and Nf1+/+ littermates were assigned to observation (control group) or to receive treatment with either etoposide or cyclophosphamide beginning at 6 to 10 weeks of age. These agents were selected because they are used widely in human cancer therapy. Treated mice received a single 6-week course of 100 mg/kg/wk of either agent, a schedule which approaches the maximally tolerated doses. Cyclophosphamide (CY) was administered by intraperitoneal injection whereas etoposide was administered through an orogastric tube.

CBCs with white blood cell (WBC) differentials were performed every 3 months in mice that appeared well, and whenever a mouse showed signs of systemic illness. The CBC was repeated immediately whenever the WBC count was >20,000/μL. All mice that appeared moribund and animals with WBC counts >20,000/μL on two consecutive determinations were killed, the spleens were weighed, and hematopoietic tissues were collected for morphologic and genetic analysis.

Nf1 genotyping and LOH analysis. Genomic DNA was prepared from tail clippings or from hematopoietic tissues (spleen or bone marrow) by standard procedures.22 Nf1 genotypes and loss of heterozygosity were determined by digesting DNA samples with NcoI + HindIII followed by gel electrophoresis, blotting to nylon membranes, and hybridization with an NcoI-PstI fragment from intron 31 of Nf1 as described previously.16 LOH was scored by comparing the relative intensities of restriction fragments derived from paired normal and leukemic tissues.

LOH analysis with microsatellite markers. These procedures have been described in detail.11 Briefly, DNA samples were amplified in a DNA Thermocycle Machine (Perkin Elmer Cetus, Norwalk, CT). Polymerase chain reaction (PCR) was performed in reaction mixtures that include 0.66 μmol/L of respective 3′ and 5′ primers, 100 ng of target genomic DNA, 1 U of Taq polymerase (AmpliTaq; PE Applied Biosystems, Foster City, CA), and 0.4 μmol/L final concentrations of deoxynucleotides in a final reaction volume of 25 μL. The forward primer was kinase-labeled with γ-32P adenosine triphosphate (ATP). Labeled PCR products were separated on (6 mol/L urea, 8% polyacrylamide) sequencing-type gels and run at 60 to 80 W constant power for 2 to 4 hours. The gels were dried, placed in Saran wrap (Dow Brands L.P., Indianapolis, IN), and exposed to x-ray film at −70°C. The polymorphic markers tested included D18Mit55, D18Mit13, and D13Mit13, which are syntenic to human 9q31 and D6Mit48, D5Mit40, and D12Mit64, which are syntenic to genes within human 7q22-31.

Cytogenetic analysis and fluorescence in situ hybridization (FISH). A trypsin-Giemsa banding technique was used to analyze cells from bone marrow and spleen. Metaphase cells from short-term (24 to 72 hours) unstimulated cultures were examined. Ten metaphase cells were examined each from the bone marrow and spleen cultures for each mouse. Chromosomes were identified using the standardized mouse karyotype as described by Cowell.23 FISH was performed as described previously.24 Briefly, a biotin-labeled Nf1 probe was prepared by nick-translation using Bio-16-dUTP (Enzo Diagnostics). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4,6-diamidino-2-phenyldiido-hydriodochloride (DAPI). Images were obtained using a Zeiss Axioshot microscope coupled to a cooled charge coupled device (CCD) camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NU200, Photometrics Inc, Phoenix, AZ and NIH Image 1.57, National Institutes of Health, Bethesda, MD). The Nf1 probe used for FISH was a 10.6 kb genomic lambda clone containing exon 31 and flanking intron sequences.

RESULTS

Leukemia in Nf1+/− and Nf1+/+ mice. Myeloid disorders developed in 4 of 101 Nf1+/+ mice, 2 of which received CY (Table 1). In contrast, myeloid malignancies were diagnosed in 14% of the untreated Nf1+/− mice (8 of 58), in 25% of the etoposide-treated animals (8 of 32), and in 38% (14 of 37) of the mice assigned to the CY group (Table 1). Kaplan-Meier plots comparing disease incidence over time in Nf1+/+ and Nf1+/− mice that received no treatment, etoposide, or CY are shown in Fig 1. Nf1+/− mice that received either drug had a significantly higher rate of disease than wild-type mice treated in parallel (Fig 1). Treated and untreated Nf1+/+ mice were also compared to ascertain the relative contributions of Nf1 genotype and chemotherapy exposure to leukemia susceptibility. This analysis showed that the incidence of disease was significantly higher, and the latency period shorter, in the Nf1+/− mice that received CY (0.004 v untreated Nf1+/− mice by pairwise logrank statistics), but not in the etoposide group (P = .2 v the untreated group). The in vivo leukemogenic effect of CY was restricted to Nf1+/− mice as Nf1+/+ animals in the control and CY-treated groups had similar rates of leukemia (Table 1). The incidence of leukemia was higher in Nf1+/− mice from the inbred 129/Sv background than in 129/Sv×C57BL/6 animals (Table 1), although these differences did not achieve statistical significance.

A myeloproliferative phenotype was observed in most diseased mice that was similar in control and chemotherapy-treated animals. My MPD was characterized by elevated peripheral blood leukocyte counts with a high percentage of mature neutrophils and monocytes (Fig 2). The mean WBC count was 31,000/μL (range, 20,000 to 98,000), and the mean myeloid cell count was 28,000/μL (range, 14,000 to 88,000). Blood smears showed a variable degree of myeloid differentiation with some containing greater than 80% mature neutrophils and others showing 30% to 40% monocytes and monocytoid

<p>| Table 1. Incidence of Leukemia in Nf1 Mice |</p>
<table>
<thead>
<tr>
<th>Genotype and Treatment</th>
<th>Genotype</th>
<th>No. of Mice</th>
<th>No (%) with Leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv</td>
<td>None</td>
<td>Nf1+/+</td>
<td>31 (6%)</td>
</tr>
<tr>
<td></td>
<td>Etoposide</td>
<td>Nf1+/+</td>
<td>46 (8%)</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>Nf1+/+</td>
<td>26 (0%)</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>Nf1+/+</td>
<td>32 (8%)</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>Nf1+/+</td>
<td>5 (0%)</td>
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<tr>
<td></td>
<td>CY</td>
<td>Nf1+/+</td>
<td>12 (7%)</td>
</tr>
<tr>
<td>129/Sv × C57BL/6</td>
<td>None</td>
<td>Nf1+/+</td>
<td>14 (0%)</td>
</tr>
<tr>
<td></td>
<td>Etoposide</td>
<td>Nf1+/+</td>
<td>12 (0%)</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>Nf1+/+</td>
<td>25 (2%)</td>
</tr>
<tr>
<td></td>
<td>CY</td>
<td>Nf1+/+</td>
<td>25 (7%)</td>
</tr>
</tbody>
</table>
cells. Some smears showed rare blasts. Platelet counts and hemoglobin values were normal in mice with MPD and immature erythroid lineage cells were not seen in the peripheral blood. There was no consistent relationship between treatment group, WBC count, and the degree of myeloid maturation visible on blood smears. The bone marrows of mice with MPD showed an overwhelming predominance of myeloid cells with a shift toward immature elements, and sections of the spleen showed expansion of red pulp with infiltration of myeloid cells at various stages of differentiation admixed with areas of erythropoiesis (Fig 2). This MPD is similar to the JMML-like disorder that arises after adoptive transfer of Nf1<sup>−/−</sup> fetal liver cells into irradiated recipient mice. A disease phenotype more consistent with acute leukemia was seen in one CY-treated Nf1<sup>+/−</sup> mouse and in one mouse that received etoposide. Both animals had WBC counts >150,000/µL with large numbers of blasts and few mature neutrophils in the peripheral blood. The CY-treated mouse also had anemia (hemoglobin level, 5.7 g/dL) and thrombocytopenia.

**Laboratory investigation of murine leukemias.** LOH at Nf1 correlated with clinical evidence of leukemia in Nf1<sup>+/−</sup> mice (Table 2) and this invariably involved loss of the wild-type Nf1 allele (Fig 3). Within the CY-treated group, leukemic cells from 129/Sv × C57BL/6 mice showed a much lower incidence of LOH than cells from 129/Sv animals (Table 2). Both animals with evidence of acute leukemia had LOH in hematopoietic tissues. In mice with MPD, LOH was not consistently associated with higher leukocyte counts or with increased numbers of immature myeloid cells. Unexpectedly, we detected LOH at sacrifice in the hematopoietic tissues of 18% of mice that did not fulfill the criteria used to diagnose leukemia. Most of these animals appeared well and WBC counts <10,000/µL and absence of prominent myeloid infiltrates in splenic sections. These results implicate inactivation of Nf1 as an early event that confers an in vivo proliferative advantage upon a clone of cells, but also suggest that additional mutations are required to produce the characteristic MPD. LOH in the absence of leukemia was much more common in 129/Sv mice than in 129/Sv × C57BL/6 animals (Table 2). Among mice without leukemia, LOH was relatively common in control animals but infrequent in the etoposide-treated cohort (Table 2).

Cytogenetic analysis of bone marrow and spleen cells from 6 mice with MPD (5 CY-treated mice and 1 from the etoposide group) revealed a normal karyotype (Fig 4A). To ascertain if LOH on Southern blots was associated with submicroscopic deletions of Nf1 or with duplication of the mutant allele, we used a genomic Nf1 probe from the disrupted segment of the gene to perform FISH analysis of hematopoietic cells from 3 of these 6 mice. FISH showed 2 structural copies of the Nf1 gene in each case (Fig 4B). We also used six polymorphic microsatellite markers to examine bone marrow DNA from mice with t-ML for LOH at loci syntenic to regions of human chromosomes 5 and 7 that are frequently deleted in humans with t-MDS and t-AML, but found none (data not shown). Similarly, Southern blot analysis of specimens from etoposide-treated mice did not show rearrangements of Mll when hybridized with a probe from the human MLL breakpoint cluster region that detects virtually all of the breakpoints in human leukemias (data not shown).

**DISCUSSION**

This study establishes an in vivo model of therapy-induced myeloid malignancies in Nf1<sup>+/−</sup> mice that will facilitate basic and translational research studies of this important clinical disorder. In human t-MDS/t-AML, frequent deletions involving chromosomes 5 and 7 have implicated loss of gene function in genotoxic-induced leukemogenesis. How alkylating agents actually cause leukemia is unknown; however, CY increases the frequency of somatic inactivation of target genes in a variety of
assays. In contrast, leukemias that arise after exposure to topoisomerase II inhibitors are associated with recurring chromosomal translocations involving the *MLL* gene that result in the production of dominantly acting chimeric proteins. If the leukemias that develop after treatment with alkylating agents predominately involves the inactivation of specific target genes, it is possible that some human patients who develop t-ML after alkylator-based chemotherapeutic regimens are highly susceptible because of germline mutations of undiscovered tumor-suppressor genes that, like *NF1*, restrain the growth of immature myeloid cells.

We used clinical criteria to diagnose leukemia because the bone marrows of some children with NF1 who develop malignant myeloid disorders do not show LOH at *NF1*. In this study, mice with clinical evidence of MPD or AML had a threefold higher rate of LOH at *Nf1* than mice without these findings. The presence of somatic LOH in hematopoietic tissues supports the clonal nature of these myeloid disorders. MPDs

<table>
<thead>
<tr>
<th>Genotype and Treatment</th>
<th>No. of Mice</th>
<th>No. With LOH/No. With Leukemia</th>
<th>No. With LOH/No. Without Leukemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>129/Sv None</td>
<td>46</td>
<td>5/8 (62%)</td>
<td>10/38 (26%)</td>
</tr>
<tr>
<td>Etoposide</td>
<td>32</td>
<td>7/8 (87%)</td>
<td>1/24 (4%)</td>
</tr>
<tr>
<td>CY</td>
<td>12</td>
<td>5/7 (71%)</td>
<td>4/5 (80%)</td>
</tr>
<tr>
<td>129/Sv × C57BL/6 None</td>
<td>12</td>
<td>0/0 (0%)</td>
<td>1/12 (8%)</td>
</tr>
<tr>
<td>CY</td>
<td>25</td>
<td>2/7 (28%)</td>
<td>1/18 (5%)</td>
</tr>
<tr>
<td>All mice</td>
<td>19/30 (63%)</td>
<td>17/97 (17%)</td>
<td></td>
</tr>
</tbody>
</table>

![Fig 2. Tissue sections from CY-treated Nf1+/− mice with MPD.](image)

(A and B) Blood and bone marrow smears showing immature and well differentiated myeloid cells. (C) A cytocentrifuge preparation of spleen cells stained with the myeloid lineage marker nonspecific esterase demonstrates many positive cells (brown stain). (D) A spleen section shows a dense infiltrate of myeloid cells within the red pulp.

![Fig 3. Southern blot analysis of tissues from Nf1+/− mice with MPD.](image)

The 3.2-kb restriction fragment corresponds to the targeted *Nf1* allele, and the 2.4-kb band is derived from the wild-type allele. DNA extracted from the bone marrows of five mice with leukemia (L) show absence or a marked reduction in the wild-type *Nf1* allele compared to paired tail (T) DNA specimens.
with and without LOH had similar features to the myeloid disorder that emerges after adoptive transfer of Nf1−/− fetal liver cells into irradiated recipient mice.17 Together with the extraordinary increase in the incidence of leukemia in Nf1+/− versus Nf1+/+ animals, these observations provide evidence that inactivation of Nf1 is a central event in leukemogenesis even in the absence of LOH. If this is true, it is likely the wild-type Nf1 allele is inactivated in bone marrows without LOH by subtle somatic mutations. An alternative consideration is that some Nf1+/− mice develop myeloid malignancies through a genetic pathway that does not involve biallelic inactivation of Nf1, as has recently been shown for a subset of tumors from heterozygous p53 knockout mice.26 Experiments using techniques that can identify point mutations will be required to distinguish between these possibilities. Nf1 is a very large gene, and protein truncation has proven to be the most efficient method for detecting subtle mutations in normal and leukemic cells from Nf1 patients.20,27,28

Adoptive transfer of Nf1−/− fetal liver cells into irradiated mice consistently induces a MPD with features of JMML.17 Inasmuch as these data suggested that inactivation of Nf1 in early hematopoietic cells might be both necessary and sufficient to induce clinical disease, we were surprised to detect LOH at Nf1 in hematopoietic tissues from 18% of Nf1+/− mice with normal WBC counts. This idea that genetic alterations in addition to inactivation of Nf1 are required for clinical disease is consistent with the relatively long latency between CY exposure and the onset of t-ML in Nf1+/− mice (Fig 1). Cooperating somatic mutations such as bone marrow monosomy 7 and epigenetic events have also been identified in human Nf1-associated myeloid disorders.21,29 It will be of interest to determine if LOH can be detected in circulating blood cells some months before the onset of leukocytosis and splenomegaly in Nf1+/− mice.

We did not inject bone marrow cells from Nf1+/+− mice that acquired myeloid disorders associated with LOH into secondary hosts. In our hands, transferring marrow from recipients previously engrafted with Nf1−/− fetal liver cells consistently induces MPD in irradiated, but not in unirradiated, mice (data not shown). In an interesting experiment, Largaespada et al17 crossed a mutant Nf1 allele into the BXH2 line of mice in which a leukemogenic retrovirus is transmitted vertically from mother to pups. They observed preferential viral integration into the wild-type Nf1 allele, shortened latency, and a change in disease phenotype from MPD to AML.17 Their finding of other somatically acquired leukemia-specific viral integrations within these clones implicated alterations in addition to inactivation of Nf1 in progression from MPD to AML. Adoptive transfer into secondary recipients provides a way of further characterizing therapy-induced myeloid disorders arising in Nf1 mice and may be especially informative in rare cases that show features of acute leukemia.

LOH at Nf1 and clinical leukemia were more common in homozygous 129/Sv mice than in 129/Sv × C57BL/6 animals. This was true in both control and CY-treated mice. Thus, 129/Sv hematopoietic cells are unexpectedly prone to spontaneously undergo LOH at Nf1 followed by clonal expansion. Rates of cancer in the F1 progeny of crosses between two inbred mouse strains often correlate poorly with parental rates and may be higher, lower, or unchanged.36 The net effect of our having assigned disproportionate numbers of 129/Sv × C57BL/6 mice to the CY group is to understate the magnitude of the leukemogenic effect of this agent. CY-treated mice showed a higher incidence of clinical leukemia than the control group irrespective of genotype (58% vs 17% in strain 129/Sv and 28% vs 0% in strain 129/Sv × C57BL/6; Table 1).

LOH was less frequent in CY-treated 129/Sv × C57BL/6 mice with MPD than in any of the 129/Sv cohorts. This low incidence suggests that the mechanism of Nf1 inactivation in 129/Sv × C57BL/6 hematopoietic cells involves subtle alkylator-induced mutations rather than loss of the wild-type allele. Consistent with this, Shoemaker et al11 recently identified somatic Apc point mutations caused by transitions or transversions in intestinal tumors from multiple intestinal neoplasia (Min) mice that had been exposed to the alkylating agent N-ethyl-N-nitrosourea (ENU). Interestingly, other tumors from this ENU-exposed cohort showed LOH at Apc. Taken together with our data from CY-treated mice, these data suggest that mechanisms of alkylator-induced tumor suppressor gene inactivation in colonic and hematopoietic cells include somatic rearrangements that result in LOH as well as subtle intragenic events.
Intestinal tumors that spontaneously arise in Min mice show LOH at Apc with apparent loss of an entire chromosome 18 homolog. However, in a line of mice that carried mutations of the Apc and Dpc4 tumor suppressor genes in cis, intestinal tumorigenesis was associated with apparent loss of one entire chromosome homolog with duplication of the mutant chromosome. Consistent with this, FISH analysis of murine leukemias with LOH showed an Nf1 allele on each chromosome 11 homolog. Although deletion of the chromosome containing the normal tumor suppressor gene allele followed by duplication of the mutant homolog has been proposed as a likely underlying mechanism, other models are also plausible. Mitotic nondisjunction resulting in two copies of the mutant homolog might occur first, with subsequent loss of the normal chromosome. Alternatively, the DNA segment that contains the normal allele might be replaced by a homologous segment from the mutant chromosome by a double mitotic recombination event, as has been reported in a human NFI-associated leukemia.

Haran-Ghera et al. previously observed a weak leukemogenic effect of multiple doses of CY when this agent was administered with radiation and dexamethasone to SIL/J mice, a strain that is susceptible to radiation-induced AML. However, CY did not induce leukemia in the absence of radiation, and only cooperated with radiation when it was combined with dexamethasone. In contrast, we have developed a murine model of t-ML based on clinical observations in NFI patients in which CY alone efficiently induces myeloid leukemia in Nf1−− mice.

Our data provide direct experimental evidence that exposure to a commonly used cancer chemotherapeutic agent can cooperate with a genetic predisposition in the development of myeloid malignancies. Although human patients with t-MDS/t-AML frequently show peripheral blood cytopenias when they seek medical attention, their bone marrows are hypercellular and the disease typically evolves into a frankly proliferative phase with time. Similarly, Nf1−− mice only exhibit overproliferation of myeloid cells months after exposure to CY. As in humans, LOH in murine hematopoietic cells is associated with a copy of the mutant Nf1 allele on each chromosomal homolog. The relevance of this model to human leukemia is further suggested by the presence of genetic alterations that deregulate Ras signaling in many human myeloid leukemias and by the finding of activating Ras mutations in the bone marrows of some patients with t-AML. Although our data implicate mutations of genes in addition to Nf1 in murine leukemogenesis are also consistent with observations in human patients, we did not detect LOH with polymorphic markers from regions of the murine genome that are syntenic to human 5q31 and 7q22. There are a number of potential explanations for these findings, including: (1) the probes used might be some distance from the critical murine loci, (2) the relevant murine genes may be inactivated by somatic mutations which do not result in LOH, (3) loss of DNA sequences syntenic to human 5q31 and 7q22 could be late events in progression of MPD to AML that had not occurred by the time of sacrifice, and/or (4) a different spectrum of cooperating genes might be mutated in human and murine leukemias. The nature of the alterations that are involved in alkylator-related leukemias awaits identification of additional target genes in both species.

This novel model provides a rigorous in vivo system to address a number of important (and in some cases controversial) issues in therapy-related myeloid disorders including the relative leukemogenic potential of different alkylating agents, the role of dose intensity, and the additive effects (if any) of alkylating agents and external beam radiotherapy. Furthermore, molecular analysis at Nf1 may elucidate the mechanistic basis of genetic damage induced by specific alkylating agents in immature hematopoietic cells. Nf1−− knockout mice will also be useful for testing the utility of surrogate markers of gene mutation such as inactivation of Hprt to ascertain if exposure to specific mutagens portends an elevated risk of leukemia and to investigate chemopreventive strategies. Finally, these results have implications for the care of individuals with NFI who develop neoplasms, because they suggest that alkylator-based regimens should be avoided whenever possible.

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