The inv(16) Cooperates with ARF Haploinsufficiency to Induce Acute Myeloid Leukemia*

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The inv(16) is one of the most frequent chromosomal translocations associated with acute myeloid leukemia (AML) and creates a chimeric fusion protein consisting of most of the runt-related X1 co-factor, core binding factor β fused to the smooth muscle myosin heavy chain MYH11. Expression of the ARF tumor suppressor is regulated by runt-related X1, suggesting that the inv(16) fusion protein (IFP) may repress ARF expression. We established a murine bone marrow transplant model of the inv(16) in which wild type, Arf+/−, and Arf−/− bone marrow were engineered to express the IFP. IFP expression was sufficient to induce a myelomonocytic AML even when expressed in wild type bone marrow, yet removal of only a single allele of Arf greatly accelerated the disease, indicating that Arf is haploinsufficient for the induction of AML in the presence of the inv(16).

The gene encoding the runt-related-X1 (RUNX1, also known as acute myeloid leukemia 1 (AML1)) transcription factor is one of the most frequently mutated genes in human B-cell acute lymphoblastic leukemia (ALL) and AML. RUNX1 is inactivated by bi-allelic mutations in cases of AML with very immature blasts (1, 2) and is also disrupted by the t(12;21), which is the most frequent translocation associated with childhood B-cell ALL (3). Furthermore, RUNX1 is also targeted by many chromosomal translocations in AML, including t(3;21), t(16;21), and t(8;21), and the latter is one of the most frequent translocations (12–15%) in AML (4). Finally, the functions of RUNX1 are also indirectly compromised by the inv(16), which disrupts the gene encoding the RUNX1 co-factor, core binding factor β (CBFβ). CBFβ is a small protein that allosterically regulates the ability of RUNX1 to bind to DNA and that blocks the ubiquitin-mediated degradation of RUNX1 (6–8). The inv(16) occurs in roughly 8% of AML patients and encodes a fusion protein that contains most of CBFβ fused to a smooth muscle myosin heavy chain (MYH11) (4, 5). (For simplicity, we will refer to the inv(16) fusion protein as the “IFP.”)

Recent insights suggest that RUNX1 regulates the transcription of genes that can predispose hematopoietic stem cells or progenitor cells to oncogenic transformation. One such target is the p14ARF (alternative reading frame) tumor suppressor, which regulates the p53-dependent oncogene checkpoint by antagonizing MDM2 functions, thereby stabilizing and activating p53 (9–13). In murine cells, p19ARF (the murine homolog of human p14ARF, referred to here collectively as ARF) levels increase in response to various oncoproteins, including v-Abl, c-Myc, Ras, E2F-1, and E1A (11, 14–17). Thus, loss of ARF impairs p53-mediated growth arrest and/or apoptosis in response to oncogenes.

The development of an inv(16) animal model in which to test the mechanism of action of the IFP has been extremely difficult. Previous murine models of the inv(16) have involved the expression of the IFP coupled with either ENU mutagenesis, retroviral insertional mutagenesis (18, 19), the removal of two tumor suppressors (20), or the co-expression of oncogenes (20, 21). However, only the use of chimeric mice carrying a “knock-in” inv(16) allele plus random mutagenesis yielded an AML that mimicked the human disease (18, 19).

Given that RUNX1 and RUNX1-ETO (Eight-Twenty-One) regulate the activity of the ARF promoter (22), we reasoned that the IFP may also influence Arf expression and that this pathway would be operational in myeloid leukemogenesis. We demonstrated, in a bone marrow transplant (BMT) model, that the IFP is sufficient to induce AML and that this disease is held in check by Arf.

EXPERIMENTAL PROCEDURES

Plasmids—The MSCV-ires-GFP retroviral construct encoding the IFP was created by subcloning the entire coding region of this cDNA into the murine stem cell virus (MSCV) vector. MSCV-c-MycER was created by subcloning the c-MycER cDNA (23) into the MSCV-Puro vector.

Viral Infection of Bone Marrow-derived Cells—Bone marrow was harvested from the femurs of wild type, Arf+/−, and Arf−/− mice (24) (The Jackson Laboratory, Bar Harbor, ME). 6–8-week-old donor mice were injected 4 days before with 140 mg 5′-fluorouracil (Acrros)/kg of weight. Harvested cells were cultured in RPMI 1640 medium (Cambrex) with 10% fetal bovine serum, 10 ng/ml interleukin-6, 10 ng/ml stem cell factor, and 3% conditioned medium from Chinese hamster ovary cells expressing interleukin-3. Viral supernatants were prepared using vesicular stomatitis virus G glycoprotein (VSV-G)-pseudotyped retroviruses. 293-GFP2 cells were transfected using Polyfect (Qiagen). Viral supernatants were pooled, centrifuged at 20,000 × g for 2 h at 4 °C, and resuspended in RPMI 1640 medium plus fetal calf serum with 5 μg/ml poly-
A Murine Model for inv(16)-induced AML

bren(e (Sigma). Bone marrow cells were spin inoculated at 1800 x g for 1.5 h. In addition, these cells were cultured together with irradiated (30 gray) virus-producing BOSC23 cells prior to transplant. Approximately 5 x 10^5 to 1 x 10^6 cells, of which 10–30% were GFP^+, were injected via the tail vein into lethally irradiated 6–8-week-old syngeneic recipient mice.

Protein/DNA Analysis—DNA from the spleens of transplanted animals was extracted using the DNeasy tissue extraction kit (Qiagen). The DNA was digested to completion using the indicated restriction endonucleases and Southern blot analysis performed using probes to the retroviral vector (GFP) to determine the number of retroviral integration sites or a DNA fragment containing exon 1β of Arf. Immunoblot analysis was performed as previously described using antibodies directed to p19Lin (Abcam), CBFβ (25), and GADPH or β-actin as loading controls.

Flow Cytometry and Analysis of Leukemia—Standard three-color flow cytometry on a BD Biosciences FACSCalibur flow cytometer was used for the majority of the analysis using fluorescently labeled antibodies directed to CD117 (c-Kit), CD11b (Mac-1), Ter119, B220, CD3, CD4, and CD8 (BD Biosciences) labeled with PE and Sca-1 and Gr-1 (Caltag) labeled with PE-Cy5. The analysis of lineage-negative (PE) and Scal (PE-Cy5)- versus c-kit (APC)-positive cells was performed on a FACSaria flow cytometer. Histological analysis was performed on peripheral blood smears and on formalin-fixed paraffin-embedded sections of the liver, spleen, and bone marrow. Kaplan-Meier plots and the Log-Rank test were performed using the SPSS, version 8.0, statistical software package. The Student’s t test were used to show that the differences in the size of spleens were significant at the 0.99 confidence level.

Reporter Assays—NH3T3 or HeLa cells were transfected with the indicated amount of plasmids encoding RUNX1, the IFP, or RUNX1(L175D), which cannot bind DNA, and 1 µg of the p14^{Arf} promoter-reporter plasmid. A plasmid encoding a secreted form of alkaline phosphatase or Renilla luciferase was used as an internal control plasmid for transfection efficiency, empty pCMV5 was used as a vector control, and pBlueScript was used to balance the DNA content in the transfections. 40 h after transfection, cell lysates were assayed for firefly luciferase activity using the luciferase assay reagent (Promega), and secreted alkaline phosphatase activity in the culture medium was used to normalize transfection efficiency.

RESULTS

The IFP Cooperates with Arf Heterozygosity to Induce Myeloid Leukemia—Previous murine models of the inv(16) have relied on expression of the IFP coupled with mutagenesis (18, 19), the removal of the Arf and p16\^{ink4a} tumor suppressors (20), or the co-expression of oncogenes (20, 21). However, only the use of chimeric mice created from embryonic stem cells containing MLYH11 inserted into the CBFβ locus, coupled with either ENU or retroviral insertional mutagenesis, has yielded an acute myeloid leukemia (18, 19). The observed cooperation between the IFP and deletion of the Arf/p16 locus was intriguing, given that Arf is a target for regulation by RUNX1 (22). Because this deletion is manifest in the germ line, the deletion of the Arf/p16 locus likely cooperates with the IFP, because the marrow of 6–8-week-old mice contains mutations that accumulate in the absence of these tumor suppressors. However, this model yielded ALL (20) rather than AML.

This presupposes that the IFP does not completely silence Arf expression and that deletion of Arf (but not Arf/p16) allows the accumulation of mutations that would accrue in the presence of the inv(16).

To express the IFP in this model system, we subcloned the gene expressing the 611-amino-acid isoform of the inv(16) into a MSCV recombinant retrovirus vector containing an IRES linked to GFP and transduced bone marrow-derived stem cells and progenitors from wild type. Arf\^{−/−}, and Arf\^{−/−}−/− littermates (Fig. 1). Bone marrow-derived cells were cultured in RPMI 1640 medium containing fetal calf serum supplemented with interleukin-3, interleukin-6, and stem cell factor. After spin inoculation, roughly 10–30% of the cells expressed GFP, and ~1 million cells were used to reconstitute irradiated recipient mice. Control mice transplanted with wild type, Arf\^{−/−}, or Arf\^{−/−}−/− bone marrow cells infected with the empty vector (MSCV-IRES-GFP) did not show signs of disease during the course of the study. Strikingly, within 12 weeks following adoptive transfer, more than half (5/9) of the mice transplanted with inv(16)/Arf\^{−/−}−/− bone marrow showed signs of distress and had elevated white cell counts (Fig. 1, left panel, open boxes).

Unexpectedly, by 15 weeks, 57% (8/14) of the mice reconstituted with the inv(16)/Arf\^{−/−}−/− stem cells had also developed disease (Fig. 1, left panel, open diamonds), and by 18 weeks, all recipients expressing the IFP in either Arf\^{−/−}−/− or Arf\^{−/−}−/− bone marrow had succumbed to an apparent leukemia. Statistical analysis indicated no significant difference at the 95% confidence level when comparing the outcome of IFP expressed in bone marrow from Arf\^{−/−}−/− or Arf\^{−/−}−/− mice. By contrast, mice reconstituted with IFP-expressing bone marrow from wild type mice only showed increased white blood cell counts by 6–7 months post-transplant and with only ~40% penetration (4/9 in this experiment) (Fig. 1).

Upon necropsy, all inv(16)/Arf\^{−/−}−/− and inv(16)/Arf\^{−/−}−/− mice showing outward signs of disease displayed splenomegaly (Fig. 2A), and some also had obvious hepatomegaly (data not shown). Histological sections of the spleens showed complete effacement of the splenic architecture, compared with normal spleen (Fig. 2, B and C), due to infiltration of the red pulp by immature hematopoietic cells. Histological sections of the liver demonstrated large periportal and perisinusoidal infiltrates of similar immature hematopoietic cells as compared with normal liver (Fig. 2, D and E). Immunoblot analysis of splenic extracts from these diseased mice confirmed IFP expression at levels comparable with that expressed in the inv(16)-containing cell line ME-1 (Fig. 2F). In addition, transfer of inv(16)/Arf\^{−/−}−/− and inv(16)/Arf\^{−/−}−/− leukemic blasts into secondary recipients induced a similar disease with 100%
penetrance (data not shown), suggesting that the IFP can cause the development of an aggressive leukemia and that this cooperates with haploinsufficiency or loss of Arf.

inv(16)⁺/Arf⁻/⁻ Leukemic Blasts Display Characteristics of an Immature Myelomonocytic AML—The peripheral blood of diseased inv(16)⁺/Arf⁺/⁺ and inv(16)⁺/Arf⁻/⁻ recipients contained high percentages (up to 80%) of immature white blood cells with large nuclei and a modest amount of blue cytoplasm (Fig. 3A), and touch preparations of the spleens indicated the presence of cells with a similar morphology (Fig. 3B). Many of these leukemic cells in the peripheral blood and spleen contained basophilic granules (Fig. 3, A and B), suggesting a myeloid origin. In addition, we noted that the granules observed by Wright-Giemsa staining were similar to those observed in the abnormal eosinophils found in inv(16)-containing patients and also that some blasts contained cytoplasmic vacuoles (Fig. 3, A and B) that are typical of monocytes.

To confirm the myeloid nature of the leukemic blasts, we immunophenotyped these cells in both the bone marrow and spleens by flow cytometry (Fig. 3C shows representative data from bone marrow). Regardless of their Arf status and consistent with their immature morphology, the GFP⁺ IFP-expressing leukemic cells either lacked or displayed low expression of the lineage markers typical of more mature lymphocytes, erythroid, or myeloid cells (CD45/B220, Ter119, CD3, CD4, CD8, Gr-1, Mac-1), anti-c-Kit (c-Kit-APC), and anti-Sca-1 (ScaI-PECy5) and analyzed by flow cytometry. GFP⁺/Lin⁻ cells (encircled in left panels) were segregated and plotted as c-Kit versus Sca-1 (right panels).
Moreover, this phenotype matched the phenotype of the AML induced the hallmarks of the human inv(16)-related myelomonocytic AML. Vacuoles (Fig. 3, B), a number of cells with some monocytic features (e.g. the presence of vacuoles) (Fig. 3, A and B), this appeared to be an AML that had some of the hallmarks of the human inv(16)-related myelomonocytic AML. Moreover, this phenotype matched the phenotype of the AML induced in chimeric mice derived from injection of embryonic stem cells containing a knock-in allele that recreates the inv(16) at the CBFB locus coupled with ENU mutagenesis (18).

Southern blot analysis using genomic DNA extracted from the enlarged spleens of mice expressing the IFP was used to determine whether the leukemia observed were clonal. In most cases, only 1–2 integrations were observed, suggesting that the AML induced by the IFP was indeed clonal in nature (Fig. 4 shows a representative example). Thus, this inv(16)-induced disease fulfills all of the characteristics outlined in the “Bethesda criteria” (26) to be deemed an acute myeloid leukemia.

The inv(16) Is Sufficient to Induce AML—Among mice transplanted with IFP-expressing wild type bone marrow cells, roughly 40% developed disease but with a much extended latency compared with the IFP+/Arf+/− and IFP+/Arf−/− transplants (Fig. 1B). Similar to the disease induced by the IFP in Arf+/+ or Arf−/− mice, the morphology of the leukemic blasts arising in injection wild type IFP+ expressing bone marrow recipients suggested they were also immature myeloid cells (Fig. 5A). Some of these mice displayed no outward signs of disease and were sacrificed when increases in the percentages of GFP+ cells were detected in the peripheral blood. At this juncture, most of these mice had splenomegaly but only minimal liver infiltrates (data not shown) and a lower percentage of blasts in the peripheral blood. Also, one of these mice was found to contain leukemic blasts only following termination of the tumor watch. Immunophenotyping of the bone marrow of this mouse indicated the presence of blasts that were negative for Sca-1 with low expression of the lineage markers (data not shown) but positive for c-Kit (56.7%) (Fig. 5B), consistent with the phenotype observed with the IFP+/Arf+/+ and IFP+/Arf−/− transplants (Fig. 3). However, some of the GFP+ cells were still positive for GR-1 (3.2%), Mac-1+ (13.8%), and GR-1+ /Mac-1− (8.7%) (Fig. 5B, lower right panel), suggesting that the disease in this animal was still evolving at the time of necropsy.

IFP-induced AML Is Associated with Suppression of Arf Expression—The observation that bone marrow cells derived from IFP-expressing Arf+/− mice gave rise to AML, with only a slightly delayed onset versus AML arising in inv(16)+/Arf−/− recipients, suggested that the IFP effectively suppresses the expression of the remaining wild type allele in Arf+/− myeloid progenitors and/or that the IFP expression selected for the loss of the remaining wild type Arf allele in these leukemia cells. To address this issue, we performed Southern blot analysis to define loss of heterozygosity of Arf in these tumors. Although the exon 1B probe used to detect Arf would be expected to hybridize as well or better to the wild type fragment as compared with the mutant, it consistently yielded a weaker signal in these assays and in standard genotyping using tail DNA (Fig. 6A, see left panel and control lanes marked GFP). Therefore, in the majority of these cases (>80%), one wild type allele of Arf was retained (Fig. 6A shows examples of mice with the loss (lane 1) or the retention of the wild type Arf allele). Thus, the inv(16) cooperates with haploinsufficiency of Arf to induce AML.

We also determined whether the remaining wild type allele in IFP+/ Arf+/− blasts was expressed at the protein level. Immunoblot analysis of diseased spleens in transplanted mice showed that the protein was not detectable in the vast majority of the tumors and that only one out of nine tumors analyzed had detectable levels of Arf (Fig. 6B). Likewise, in the two tumors derived from IFP+/Arf+/− bone marrow that could be analyzed, Arf was detectable but only at low levels (Fig. 6B, right panel), as compared with the levels present in murine erythroleukemia cells or myeloid progenitor cells expressing c-Myc (Fig. 6B, bottom panel). Therefore, suppression of Arf expression is a common event in this mouse model of inv(16)-induced AML.

Given the suppression of Arf expression in the leukemic samples, we tested whether the IFP also regulates the p14ARF+ promoter in vitro using two classical model systems that have been used extensively to test the function of RUNX1 and the inv(16). RUNX1 activates transcription in HeLa cells but represses transcription in NIH3T3 cells (27, 28). Therefore, we tested the action of the IFP in both of these cell types. In HeLa cells, RUNX1 robustly activated ARF, and this activity was dependent
FIGURE 6. IFP-induced AML is associated with suppression of Arf expression. A, Arf status was analyzed by Southern blots of genomic DNA extracted from the spleens of IFP+/-Arf +/- BMT recipient mice (middle panel) and probed with radiolabeled Arf exon 1.β. The figure shows representative samples containing deletion and retention of the wild type allele. The left panel is control DNA from wild type, heterozygous, and null mice, and the right panel shows splenic DNA from leukemic mice transplanted with wild type bone marrow expressing the IFP. In the middle panel, the samples labeled GFP show DNA from mice transplanted with bone marrow from heterozygous mice infected with control virus expressing only GFP. B, Immunoblot analysis of protein extracts from the spleen of IFP+/-Arf +/- mice. Murine embryonic fibroblasts (MEF) from wild type (passage 14) or Arf +/- mice and murine erythroblastic leukemia cells were used as positive and negative controls for Arf expression. GAPDH was used as a loading control. As a further control for the induction of Arf in hematopoietic progenitor cells, primary myeloid progenitor cells from the fetal livers of mice were infected with retroviruses expressing only puromycin or expressing a c-Myc-estrogen receptor fusion protein. These cells were selected for puromycin resistance, and nuclear translocation of c-Myc-ER was induced by the addition of 4-hydroxytamoxifen. Protein extracts were analyzed by immunoblot 16 h later. C, the IFP represses the p14ARF promoter. HeLa or NIH3T3 cells were co-transfected with a p14ARF-firefly luciferase reporter-promoter (22) and RUNX1, a DNA binding-deficient mutant of RUNX1 (L175D), or the IFP expression constructs. Luciferase light units were corrected for transfection efficiency using a plasmid expressing a secreted alkaline phosphatase or Renilla luciferase. A representative Western blot from HeLa cells shows expression of RUNX1 (R) and the L175D mutant (L) in the absence or presence of the IFP (I) in the far right panel. Vec., vector; Mut, mutant; WT, wild type; RLU, relative light units. MWM, molecular weight marker.
A Murine Model for inv(16)-induced AML

on its ability to bind to DNA, as the L175D mutant that could not bind to DNA or to CBFβ (29) had no activity (Fig. 6C, top panels). When co-expressed with RUNX1(L175D), the IFP had little or no effect on ARF transcription (Fig. 6C). However, co-expression of the IFP and RUNX1 in HeLa cells impaired RUNX1-mediated activation (Fig. 6C). In NIH3T3 cells, RUNX1 repressed the ARF promoter as expected, which was also dependent on its ability to bind to DNA (Fig. 6C, lower panels). When the IFP was co-expressed with RUNX1, these factors cooperated to further repress the ARF promoter. However, co-expression of the IFP and RUNX1(L175D) had no effect on ARF transcription. Immunoblot analysis confirmed that the RUNX1(L175D) mutant was expressed at similar levels as wild type RUNX1 (Fig. 6C, right panel). In this analysis, neither endogenous RUNX1 (Fig. 6C) nor CBFβ (data not shown) were detected. Although the experiment in HeLa cells does not distinguish whether the IFP acts at the ARF promoter or simply impairs the ability of RUNX1 to bind to DNA, the cooperation between the IFP and RUNX1 indicates that this repression requires the ability of RUNX1 to bind to DNA.

**DISCUSSION**

The establishment of murine models of inv(16) AML is critical for understanding the molecular basis of how this chromosomal translocation sets the stage for the development of acute myeloid leukemia. The ARF tumor suppressor is repressed by the t(8;21) fusion protein, so we predicted that ARF would also be targeted by the IFP. Consistent with this possibility, we found that haploinsufficiency of ARF dramatically accelerated the course and penetrance of AML caused by the IFP and that ARF inactivation in this model does not occur through the deletion of the remaining wild type ARF allele, as seen in other scenarios (11, 30), but rather through reducing the expression of ARF.

Although the observed ARF haploinsufficiency may appear counterintuitive when it is a target for repression, similar effects are observed with other oncogenes that incompletely suppress the expression of tumor suppressors. For example, the cyclin-dependent kinase inhibitor p27 is transcriptionally repressed and/or degraded in response to expression of c-Myc (31–33), yet deletion of p27 cooperates with c-Myc expression in tumorigenesis (34). The cooperation between ARF haploinsufficiency and expression of the IFP is consistent with incomplete transcriptional suppression of ARF by the IFP. Thus, removal of one allele of ARF cooperates with the IFP to reduce the threshold of ARF to functionally inactivate the tumor suppressive functions of ARF (Figs. 1 and 6). Alternatively, IFP expression may select for epigenetic silencing of ARF, although it is unclear how removal of one allele of ARF would stimulate this process.

ARF expression is very low in normal cells but can be dramatically activated upon the expression of oncogenes such as c-Myc (11, 30) (Fig. 6B). This makes the interpretation of the levels of ARF mRNA in leukemic samples difficult, where the nature of the cooperating oncogene(s) is largely unknown. The levels of ARF mRNA in inv(16)-containing samples did not appear to be as consistently low as in samples containing the t(8;21) (22), but the examination of the expression of genes in AML samples is an end point analysis without control cells that phenotypically match the leukemic blasts. Nevertheless, the inv(16) samples displayed relatively low levels of ARF mRNA (22).

Our studies have also established that the IFP is sufficient to stimulate AML, although with an extended latency that would allow for other mutations to accumulate (Fig. 1). In addition to a vector control (Fig. 1), we have also expressed CBFβ in a similar fashion. Enforced expression of CBFβ failed to cause leukemia, even though it was expressed in the majority of the hematopoietic cells. The action of the IFP contrasts with the loss-of-function phenotype of the conditional knock-out of Runx1 in adult hematopoietic stem cells, which failed to develop leukemia (35, 36). Thus, the inv(16) and other translocations involving RUNX1 generate gain-of-function mutations of the RUNX1-CBFβ transcription complex. Indeed, the t(8;21) and (12;21) are dominant repressors of RUNX1 transcription targets (37–39). Similarly, the IFP cooperates with RUNX1 to repress transcription (Fig. 6C), which is consistent with observations that the IFP associates with transcriptional co-repressors (40), that it is nuclear in inv(16)-containing leukemic blasts and ME-1 cells (derived from an inv(16)-patient sample), and that essentially all of the RUNX1 is found in the nucleus in ME-1 cells (41, 42).

Previous murine models of the inv(16) relied on the use of chimeric mice carrying a knock-in inv(16) allele coupled with either ENU or retroviral insertional mutagenesis to yield an AML that mimics the human disease (18, 19). By contrast, other retroviral models have yielded predominantly lymphoid disease (20). However, we have observed the induction of an AML that appears identical to that derived from the knock-in/mutagenesis model upon expression of only the IFP. One distinction in the current model is that, prior to the development of leukemia, we did not observe impaired myeloid differentiation, but similar to the knock-in model (18), there was impaired B-cell differentiation and a nearly complete block in T-cell development. This may be due to different levels of expression of the IFP, as the retroviral system produces only low levels of the fusion protein during these early stages. Therefore, both leukemogenic and developmental phenotypes may be dissected using this system. In addition, this model may provide an important scaffold to test novel therapeutics that are currently being developed to tackle inv(16)-induced AML.

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