Consistent Up-regulation of Stat3 Independently of Jak2 Mutations in a New Murine Model of Essential Thrombocythemia

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Abstract

Janus-activated kinase 2 (JAK2) mutations are common in myeloproliferative disorders; however, although they are detected in virtually all polycythemia vera patients, they are found in ~50% of essential thrombocythemia (ET) patients, suggesting that converging pathways/abnormalities underlie the onset of ET. Recently, the chromosomal translocation 3;21, leading to the fusion gene AML1/MLD1/EVI1 (AME), was observed in an ET patient. After we forced the expression of AME in the bone marrow (BM) of C57BL/6j mice, all the reconstituted mice died of a disease with symptoms similar to ET with a latency of 8 to 16 months. Peripheral blood smears consistently showed an elevated number of dysplastic platelets with anisocytosis, degranulation, and giant size. Although the AME-positive mice did not harbor Jak2 mutations, the BM of most of them had significantly higher levels of activated Stat3 than the controls. With combined biochemical and biological assays we found that AME binds to the Stat3 promoter leading to its up-regulation. Signal transducers and activators of transcription 3 (STAT3) analysis of a small group of ET patients shows that in about half of the patients, there is STAT3 hyperactivation independently of Jak2 mutations, suggesting that the hyperactivation of STAT3 by Jak2 mutations or promoter activation may be a critical step in development of ET. [Cancer Res 2009;69(1):262–71]

Introduction

According to the WHO, essential thrombocythemia (ET) is classified as one of the six subcategories of myeloproliferative diseases (MPD), including chronic myelogenous leukemia (CML), polycythemia vera (PV), and idiopathic myelofibrosis (IMF), as well as the less frequent chronic neutrophilic leukemia and chronic eosinophilic leukemia (1). These hematopoietic malignancies arise from acquired multipotent stem cell alterations (2) resulting in overproduction of one or more myeloid cell lineages in the bone marrow (BM). In contrast to myelodysplastic syndrome (MDS), hematopoiesis in MDS is effective and the morphologic features of the myeloid cells are mature appearing.

ET was first described by Epstein and Goedel in 1934 and acknowledged as a distinct disease entity, separate from PV, in 1960 (3–5). ET is characterized as a sustained thrombocytemic state that primarily involves the megakaryocytic lineage in the BM. The major clinical complications that ET patients suffer are thrombohemorrhagic episodes. During the clinical course of their disease, ~11% to 22% of ET patients experience life-threatening major thrombotic events primarily in large arterial vessels. Bleeding episodes at presentation and during follow-up are common and usually involve the skin, gastrointestinal tract, and mucous membranes. ET patients are at high risk of progressing to acute leukemia.

Among PV, ET, and IMF, the disease-causing mutations thus far delineated are a group of activating somatic Janus-activated kinase 2 (JAK2) mutations identified in most PV patients and in ~30% to 50% of ET patients (6–8). JAK2 mediates signaling downstream of cytokine receptors after ligand-induced autophosphorylation of both receptor and kinase. The main downstream effectors of JAK2 are the signal transducers and activators of transcription (STAT) proteins. Recently, it was reported that the relatively rare (3;21)(q26;q22) was detected in a patient with ET (9). This translocation, most frequently associated with therapy-related MDS and CML (9), fuses AML1, also known as RUNX1, to MDS1/EVI1 or AME for brevity (10–12).

To clarify the role of AME in hematologic diseases, we have infected the BM progenitors with an AME-expressing retrovirus and transplanted the BM (BMT) into syngeneic recipients. Here, we show that the forced expression of AME in reconstituted mice leads to a fatal disease with a latency of 8 to 16 months. Immediately after transplantation, the peripheral blood (PB) profile and smear of all the animals show high level of abnormal platelets. These irregularities correlate with an increased bleeding time. At the time of death, the mice do not have a uniform phenotype: some of them are severely cytopenic, suggesting potential severe hemorrhaging due to intrinsic platelets dysfunction, whereas others are not cytopenic but have signs of ischemic complications. The morphologic analysis of the BM indicates normocellularity, lack of dysplasia, and the expected myeloid/erythroid ratio; however, there is evidence of megakaryocytic hyperplasia, clustering, and endosteal dislocation. We did not detect any of the reported Jak2 mutations in these animals, however a Western blot screen of the major downstream targets of Jak2 revealed a consistent hyperactivation of Stat3 in the BM of the AME-positive mice. Rather than a secondary event, this hyperactivation is directly related to AME as it is observed in AME-transfected cells lines. We used biochemical assays including reporter gene studies with a Stat3 promoter to understand the role of AME and found that AME affects the Stat3 promoter. We analyzed a small group of ET patients to determine whether the inappropriate up-regulation of Stat3 might be a critical step in the development of ET.
STAT3 could be a common event in ET patients independently of JAK2 activation. The results show that STAT3 is activated in a subgroup of the patients and suggest that the deregulation of alternative pathways that converge on STAT3 activation could be a necessary step in ET.

Materials and Methods

DNA plasmids. AME-MSCV retrovirus, AME-pCMV, and AME deletion mutants were described (13). The mouse Stat3 promoter (−1964 to +103) was amplified by genomic PCR and cloned in the promoterless luciferase reporter pGL4.20[luc2/puro] (Promega). Stat3 promoter fragments were generated using existing restriction sites. All plasmids were verified by DNA sequencing.

Cell infection and transfection. To generate infectious retrovirus particles, we transfected 20 μg of plasmid/10-cm plate in the packaging Phoenix cells with Escort V (Sigma-Aldrich). DNA-transfection of adherent cells was performed by the calcium phosphate precipitation method. For transfection of suspension cells, we used the electroporator Nucleofector II (Axama Biosystems).

Cell culture and BMT. Adherent cell lines were maintained as described (13). Murine BM progenitor cells were isolated, infected, and transplanted into lethally irradiated syngeneic recipient animals as described (14). We used C57BL/6J mice as donors and B6.SJL mice as recipients.

Patients’ materials. We received samples from Centro di Biotecnologia Avanzate in Naples (Italy). BM aspirates were obtained from 10 patients (age range, 54–76 y; median, 63) newly diagnosed with ET according to the WHO criteria. All the BM samples were hypercellular with a conserved granulocytes/erythroid ratio but an increased number of megakaryocytes.

Platelet aggregation. Preparation of mouse platelets and aggregation assay were performed as described (16). Final concentration of resuspended platelets was 3 × 10^8/mL. Platelets were allowed to rest for at least 1 h at 22 °C before use. Platelet aggregation and secretion of granule ATP were determined simultaneously in a Chronolog lumaggregometer at 37 °C with stirring (1,000 rpm) after addition of the luciferin-luciferase reagent and platelet agonists, collagen, or thrombin. Experiments were repeated at least three times.

Immunofluorescence analysis. KS62 or normal BM cells were washed 3 × with PBS and fixed in 4% formaldehyde in PBS for 20 min as described (13).

In vitro megakaryocyte differentiation. Lin- BM cells were isolated from 5- to 6-wk-old C57BL/6J mice using the Lineage Cell Depletion kit (Miltenyi Biotec GmbH). The cells were infected as described (13). After the second spinoculation, the cells were plated at a density 100,000 cells/mL in StemSpan medium (Stem Cell Technologies, Inc.) supplemented with Tpo (50 ng/mL), IL3 (10 ng/mL), IL6 (20 ng/mL), and G418 (100 μg/mL) and cultured for 20 d. Every 3 d, half of the medium in each culture was substituted with fresh medium. Cytospin preparations were prepared and analyzed daily. For immunofluorescence (IF) analysis, we used mouse unfraccionated BM cells. The cells were electroporated with an AME-expressing plasmid and cultured in StemSpan medium supplemented with Tpo (50 ng/mL) for 3 d after by IF assay.

Bleeding assay. Mice were anesthetized and positioned horizontally on a platform. After washing in PBS, the tail was transected 0.5 cm from the tip with a no. 21 surgical blade, and Whatman filter paper (Whatman International Ltd) was applied to the edge of the forming clot every 30 s, taking care not to dislodge the clot. After the bleeding stopped, the tail was immersed into PBS for 1 s to dislodge the clot and induce rebleeding from the same wound.

Reporter gene studies. The reporter gene assays were performed in NIH3T3 cells as described (13).

Western blot analysis and immunoprecipitation assays. Cells were harvested 48 h after transfection and treated as described (13). We used murine mAb M2 to the Flag epitope (Sigma), rat mAb to the HA epitope (Roche), polyclonal rabbit Ab to EVI1 (Cell Signaling Technology), polyclonal rabbit Ab to pStat3, and monoclonal murine Ab to c-Myc, Pim-1, Stat3, and Histone H1 (Santa Cruz Biotechnology). The quantification of proteins in Western blots was done by using AutoChemi Bioimaging System/LabWorks Software (UVP).

Electrophoresis mobility shift assay. AME was purified from transiently transfected 293 cells. Electrophoresis mobility shift assay (EMSA) conditions were described (17). The probe was 41 bp corresponding to the −64/−24 region of the mouse Stat3 promoter.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assay was performed with transiently transfected 293 cells as described (18). The DNA fragments were analyzed by PCR using two primers (5′-tgagaacctcgcagacagc-3′ and 5′-ggagaagccgtgct-3′) designed to amplify 176-bp of the human Stat3 promoter. As controls, we used standard human RPL30 primers designated for ChIP assay (Cell Signaling Technology) and 2 primers (5′-tgacaaccttcgccagcc-3′ and 5′-tgagaagacctgacagc-3′) designed to amplify a 193-bp fragment within the first intron of the human Stat3 gene (+2909/+3102).
Results

AME Causes a Fatal Disease in Mice

Using a recombinant MSCV retrovirus that constitutively expresses AME in murine BM cells, we generated AME-expressing C57BL/6J mice by BM transplantation. A diagram of the recombinant retrovirus is shown in Fig. 1A. The MSCV vector provides long-term low level of expression in vivo and contains the neomycin resistance selection marker suitable for in vitro studies (19). To evaluate the efficiency of infection, after infection, 50,000 BM cells were cultured in vitro with or without G418 and the number of colonies was determined. We consistently found that infection efficiency with the AME-retrovirus ranged from 14% to 17% (data not shown). As expected, given the size of the AME cDNA insert (~4.5 kb), the efficiency was about thrice lower than with the empty retrovirus (data not shown). To evaluate the extent of hematopoietic reconstitution, 4 weeks after transplantation and at time of death, we measured the percentage of donor’s marker CD45.2 by FACS. The results indicate that the engraftment ranged between 69.9% and 84.4%, and that at time of death, there was no significant expansion of the donor cells. Southern blot analysis of genomic DNA isolated from several AME-positive mice after death did not provide evidence of preferential integration sites and specific clonal expansion, indicating that the disease is very likely highly polyclonal. The expression of AME in the BM of two animals but not in the negative control was confirmed after immunoprecipitation (IP)/Western blot analysis (Fig. 1B). Aside from one mouse that developed acute megakaryoblastic leukemia and died 3.5 months post-BMT, all the remaining 12 animals survived for 8 to 16 months (Fig. 1C). Soon after BMT and until a few weeks before death, the PB counts of the mice were within the reference range aside from the platelets counts, which were abnormally high immediately after BMT and remained high during the animals’ life. At time of death (for moribund mice) or 1 to 2 weeks before death (according to the last counts for those animals that died suddenly), about half of the mice maintained WBC, RBC, and hemoglobin (Hb) counts within the reference range, whereas the remaining animals became severely cytopenic (Fig. 1D).

AME Causes Megakaryocyte and Platelet Abnormalities

Morphologic analysis—PB. To follow the development of the hematopoietic disease, periodic PB counts and smears were performed on control and AME-positive animals. The profile of PB automated counts correlated with the PB smear differential counts (200 cells counted) of AME-positive and control mice. Rare activated lymphocytes were identified in a few of the AME-positive mice (data not shown). Monocytes were mature-appearing and their relative numbers in AME-positive mice fell within the same range as control mice. The AME-positive mice showed RBC indices within the reference range during the course of the disease. However, 6 of the 12 surviving AME-positive mice developed anemia at the time of necropsy (Table 1). RBC were normochromic and normocytic in all mice; however, at time of sacrifice, five AME-positive mice had macrocytic and normochromic RBC. Minimal
Anisocytosis was most seen in patients with AME-positive mice. The most striking feature of PB smears of eight AME-positive mice relative to controls was the elevated numbers, extreme clumping, and anisocytosis of platelets (Fig. 2, A, b, and c). The difference in number of platelets compared with controls was highly significant \( (P < 0.0005) \). Giant platelets and agranular platelets (Fig. 2, A, a) were observed in the PB of several AME-positive mice during the course of disease. Morphologic analysis—BM. The BM core biopsies of the AME-positive mice were normocellular. Their most prominent feature was the marked depletion of lymphoid and myeloid elements (Fig. 2, A, d–g) were observed in the PB of several AME mice during the course of disease.

### Table 1. Hematologic variables of AME-positive mice and control mice at time of death

<table>
<thead>
<tr>
<th>Latency (mo)</th>
<th>Plt count ( (\times 10^3/\mu L) )</th>
<th>WBC count ( (\times 10^3/\mu L) )</th>
<th>RBC count ( (\times 10^6/\mu L) )</th>
<th>Hb level (g/dL)</th>
<th>Spleen weight (mg)</th>
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<tr>
<td>AME #79*</td>
<td>10.8</td>
<td>1,740</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>AME #88†</td>
<td>12.0</td>
<td>2,280</td>
<td>13.9</td>
<td>6.1</td>
<td>8.8</td>
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<tr>
<td>AME #89†</td>
<td>13.7</td>
<td>3,152</td>
<td>8.5</td>
<td>10.2</td>
<td>12.3</td>
</tr>
<tr>
<td>AME #91†</td>
<td>15.4</td>
<td>700</td>
<td>3.9</td>
<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>AME #203†</td>
<td>8.1</td>
<td>2,295</td>
<td>4.4</td>
<td>7.0</td>
<td>8.0</td>
</tr>
<tr>
<td>AME #222*</td>
<td>12.0</td>
<td>1,864</td>
<td>18.4  *</td>
<td>10.7  †</td>
<td>17.0  †</td>
</tr>
<tr>
<td>AME #251†</td>
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<td>1,890</td>
<td>1.8</td>
<td>4.3</td>
<td>5.5</td>
</tr>
<tr>
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<td>1,664</td>
<td>4.1</td>
<td>10.6</td>
<td>15.2</td>
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<tr>
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<td>2,020</td>
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<td>2.5</td>
<td>10.3</td>
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<td>1.9</td>
<td>4.0</td>
<td>6.0</td>
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<tr>
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<td>2,170</td>
<td>7.0</td>
<td>3.5</td>
<td>4.8</td>
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<td>12.24</td>
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<td>8.0</td>
<td>8.88</td>
<td>12.0</td>
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<td>13.36</td>
<td>10.16</td>
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<tr>
<td>Control #12†</td>
<td>NA</td>
<td>1,224</td>
<td>10.88</td>
<td>9.5</td>
<td>14.4</td>
</tr>
</tbody>
</table>

Note: Normal values: Plt, 766 to 1,657 \( \times 10^3/\mu L \); WBC, 3.2 to 12.7 \( \times 10^3/\mu L \); RBC, 7.0 to 10.1 \( \times 10^6/\mu L \); Hb, 11.8 to 14.9 g/dL. Abbreviations: ND, not determined; NA, not applicable; Plt, platelet.

* Died of disease.
† Data were collected 2 to 7 d before the death.
‡ Sacrificed because of moribund condition.
§ No disease.
¶ The life span of C57BL/6J mice is ~24 to 36 mo.
increase in erythroid precursor cells (Fig. 2, c and f). The lower weight of spleens in AME-positive mice was found to correlate with the shortest survival (Table 1).

**Neurologic Manifestations in AME-Positive Mice**
Before sacrifice, two of the AME-positive mice were unable to use one of their hind legs and dragged the limb when they moved, highly suggestive of hemiparesis, a neurologic manifestation clinically observed in ~12% of ET patients (22).

**Abnormal Level of PF4 but Not c-Mpl in AME-Positive Mice**
The finding of hematopoietic defects limited to megakaryocytes and platelets suggested that the expression of AME in the BM could induce a disease with features similar to ET. In ET patients, these defects are coupled with increased expression levels of PF4 (23) but normal or decreased concentrations of c-Mpl (24, 25). We used ELISA and Q-PCR to determine the level of PF4 (ELISA) in the serum and of c-Mpl and EpoR (Q-PCR) in the BM of AME-positive mice and age-matched control mice. The results indicate that at the time of death the AME-positive mice have higher levels of PF4 ($P < 0.05$; Fig. 3A). No significant difference was noted in the expression of c-Mpl and EpoR (Fig. 3B).

**Bleeding Disorders in AME-Positive Mice**
Thrombotic and hemorrhagic complications are the major reasons of morbidity in ET patients (26). We quantified the bleeding time in AME and control mice and found that as early as one month post BMT the AME-positive mice had a significantly longer bleeding and rebleeding time than the control animals ($P < 0.005$; Fig. 3C). The bleeding abnormality was maintained throughout the animals’ life (data not shown).

**Platelet Aggregation Defects in AME-Positive Mice**
Patients with ET often suffer of microvascular ischemic or thrombotic events and have shortened platelet survival together with increased platelet markers such as PF4. These defects are indicative of platelet activation and platelet-mediated thrombotic processes. The proposed concept is that platelets in ET are hypersensitive. In a recent study (27), aggregation and ATP release tests showed that 75% of MPD patients had reduced ATP release. This test seems to be one of the most direct ways to assess platelets functionality, and we tested freshly isolated platelets preparation from six AME and six control mice. It was previously reported that ET platelets respond prevalently to collagen rather than thrombin (28–31); therefore, we used two different agonists, collagen and thrombin, to stimulate platelets response. In response to thrombin, the effect of AME was not significantly different from the control (data not shown). In contrast, the collagen-induced response of AME-positive platelets in ATP secretion was impaired ($P < 0.05$; Fig. 3D). These results agree with published reports of aggregation and ATP release abnormalities of ET platelets (26).

**Expression of AME Is Sufficient to Induce Immediate Megakaryocyte Defects in vitro**
To determine whether the expression of AME is sufficient to induce early megakaryocytic defects that can be detected in vitro, we infected murine lin cells with the empty vector or AME-vector and forced them to differentiate in liquid culture along the megakaryocytic lineage by addition of Tpo, IL3, and IL6. In vector-infected cells, megakaryocytes appeared 3 to 4 days after cytokine addition, peaked at day 9, and disappeared after 17 days in liquid culture. These features are consistent with splenic atrophy, which has been reported in patients with thrombocytosis and in ET patients. The enlarged spleens (179 and 198 mg) showed a panhyperplasia with a mild relative increase in erythroid precursor cells (Fig. 2, c and f). The lower weight of spleens in AME-positive mice was found to correlate with the shortest survival (Table 1).

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culture (Fig. 4A, diamonds). In contrast, 1 day after the addition of the cytokines, we could easily observe the appearance of differentiated megakaryocyte in the AME-cell culture. The AME-megakaryocytes peaked after 3 to 4 days of culture and disappeared by day 8 to 9 (Fig. 4A, circles). In addition, in the AME-positive cell culture, there was an immediate production of platelets, which were not detected in the control cell culture (Fig. 4A and B). Taken together, these results suggest that AME forces the megakaryocytes to mature, cycle, and disappear much faster than the control cells.

Absence of Jak2 Mutations in AME-Positive Mice

Acquired Jak2 mutations are the hallmark of BCR/ABL-negative MPDs. They are detected in virtually all PV patients, in the majority of patients with IMF, and in ~30% to 50% ET patients. To determine whether anyone of the reported Jak2 mutations (V617F, K191Q, K539L, K607N, L611S, C616Y, N542E543del, I682, and D686del) was present in the AME-positive mice, we sequenced DNA extracted from the BM of four mice at the time of death. We did not detect any Jak2 mutations in these animals (data not shown).

Stat3 Is Up-regulated/Activated in AME-Positive Mice

To determine whether the JAK2/STAT signaling pathway can be altered with normal JAK2, we analyzed by Western blot the level of expression and phosphorylation of Jak2, Stat1, Stat3, and Stat5 in the BM of the AME-positive and control mice. The results indicated that Stat3 was up-regulated/activated in AME-positive mice (Supplementary Fig. S1; Fig. 5A). This alteration involved the expression of Stat3, which was overall increased compared with the controls, and its phosphorylation. We have also found Stat3 up-regulation at mRNA level (Supplementary Fig. S2). Expression of c-Myc and Pim-1, which are considered direct targets of Stat3 (32, 33), was also increased in the BM of the AME mice compared with the controls and broadly correlated with Stat3 expression. To determine whether Stat3 activation is directly related to the expression of AME, we transiently transfected normal murine hematopoietic cells with an AME-expressing plasmid and performed in vitro differentiation assay in the presence of Tpo. After 3 days in culture, AME-positive megakaryocytic cells showed up-regulation/activation of Stat3 as indicated by IF and Western blot analyses (Fig. 5B). Taken together, these data provide an...
unexpected and important link between AME and Stat3 activation.

**STAT3 Is Activated in ET Patients**

To determine whether in ET patients there is also activation of STAT3 independently of JAK2 mutations as we observed in the AME-positive mice, we analyzed the expression/activation of STAT3 in BM samples of a small group of ET patients by Western blot (Supplementary Fig. S1; Fig. 5C). We found that in about half of the ET patients there is STAT3 activation independently of JAK2 mutations.

**AME Activates the Murine Stat3 Promoter**

To determine whether AME is involved in the up-regulation of Stat3, we cloned ~2 kb of the murine promoter in a promoter-less reporter gene plasmid and generated several deletion mutants. Figure 6A (top) reports the results of reporter gene assays. In the presence of AME (black bars), there is a strong activation of the reporter gene with all the fragments of the murine Stat3 promoter aside from the smallest one encompassing the region −63/−25, suggesting that this short region, highly conserved between mouse and man, is the most critical for activation of the promoter by AME. To determine which domain of AME is involved in Stat3 activation, we tested several AME deletion mutants (Fig. 6A, bottom). The results indicated that the COOH terminus including the distal ZnF domain is necessary for promoter activation. To determine whether AME interacts with the promoter, we performed ChIP. The ChIP results (Fig. 6B) indicate that the COOH terminus of AME efficiently coprecipitates with chromatin fragments of the Stat3 promoter and suggest that the distal ZnF domain itself makes contact with the chromatin. Two negative controls for irrelevant gene RLP30 (161 bp) and for hStat3 sequence +2909/+3102 (193 bp) are also shown.

**AME Recognizes the DNA Fragment Derived from the Murine Stat3 Promoter**

To confirm that AME interacts directly with the −63/−25 region of the Stat3 promoter, we performed EMSA assay. The result (Fig. 6C) clearly shows that AME interacts to this region.

**Discussion**

In contrast to other MPDs patients, the majority of ET patients do not acquire JAK2 mutations, and ET diagnosis is therefore still dependent on clinical criteria. It is thought that ET is a heterogeneous group of very similar diseases and their biological heterogeneity is supported by the finding that each one of several molecular markers described in ET (clonal hematopoiesis, Epo-independent erythroid colony growth, PRV-1 overexpression, and reduced expression of c-Mpl) was detected only in ~50% of patients clinically diagnosed with ET (34). With the identification of activating JAK2 mutations as dominant markers in BCR/ABL-negative MPDs, several murine models have been generated with the goal of reproducing ET and understanding its pathogenesis. Surprisingly, at first several studies indicated that when expressed in a mouse BM, activated Jak2 led to PV and not ET (35–37). This dissimilarity in the effects of Jak2 mutations between a murine system and patients was explained recently as a consequence of the level of expression, and it was found that a low constitutive expression of Jak2V617F in the mouse leads to an ET-like phenotype, whereas a relatively high expression of mutated Jak2 allele leads to PV (38).

Recently, another acquired abnormality was identified in an ET patient, a t(3;21) that leads to the expression of the fusion protein AME (9). This chromosomal translocation had been previously associated with other myeloid diseases such as CML in blast crisis and aggressive MDS that quickly progress to acute myelogenous leukemia (AML; refs. 10, 11). Because the t(3;21) was cloned years ago, several studies on the role of the fusion protein in a mouse system have been published. One of the first works reported on a BMT study with BALB/c mice (39). In contrast to what we found, the BALB/c mice developed AML with a latency of 5 to 13 months. It is likely that the very aggressive phenotype described by the authors is due to the constitutive germline repression of the INK4a/ARF locus in this strain, leading to down-regulation of tumor suppressor proteins (40). Indeed, BALB/c mice are known for being more susceptible to malignancies and for generating more aggressive phenotypes when used in cancer or leukemia models than C57BL/6J animals that have an intact functional INK4a/ARF locus. It is also possible that different viral titers in this study and in the study with BALB/c mice can contribute to the phenotype differences. We have described here the phenotype of C57BL/6J mice in which expression of AME was forced in the BM. As our data show, these animals develop a disease with features and complications similar to those observed in ET patients. Of note, these animals have normal Jak2 alleles. The consistent phenotype of the mice and absence of Jak2 mutations led us to hypothesize...
that a common downstream target of Jak2 signaling, which could be activated either by Jak2 mutation or other mechanisms, is perhaps an important player in ET. Stat3 is an intermediate signaling molecule that allows ligand-induced signals from gp130-related cytokines (including IL6 and TPO) and the granulocyte colony-stimulating factor receptor to transactivate specific target genes. The maintenance of the correct level and activation of Stat3 is critical to hematopoietic cells. Injection of IL6 specifically affects megakaryopoiesis and increases platelet production in mice, without significantly disturbing WBC counts and hematocrit values (41). Later studies with a mutated gp130 unable to bind to SHP2 and SOCS3 indicated that hyperactivation of Stat3 leads to numerous hematopoietic abnormalities including thrombocytosis and splenomegaly (42). We invariably noted Stat3 up-regulation and hyperactivation in AME-positive cells. Because Jak2 was not mutated nor significantly activated in the AME-positive cells, it is possible that the basal level of Jak2 kinase activity could be sufficient by itself to activate the Stat3 protein that accumulates in the cell at a level higher than normal (43). There are also reports suggesting that, in contrast to other Stats, Stat3 does not require Y705 phosphorylation and dimerization for nuclear import/retention (44) or that unphosphorylated Stat3 activates transcription of a large subset of genes partially overlapping with the targets of P-Stat3 (45). A critical role of Stat3 in ET is supported by finding an elevated level of activation of Stat3 in approximately half of ET patients’ BM samples, whether or not JAK2 mutations had been identified, in agreement with recently published data (46). However, some published data (47) are in contradiction with our findings probably due to the different material used for the analysis (granulocytes from PB or total BM cells). We also provide here initial results indicating that AME directly activates the Stat3 promoter.

A question that must be asked is why the megakaryopoietic program seems to be the one consistently altered in the mice we generated among many pathways that control lineage commitment and which use receptors/intermediate signaling molecules that activate Stat3. The association between AME and defective megakaryopoiesis is not new and was first reported by Hirai’s group at the time when they generated a knock-in model for the fusion gene (48). Although heterozygotic embryos did not complete embryonic development and died at day 12 of fetal development, in vitro studies with fetal liver cells showed that the megakaryocytic lineage was compromised in the cells. It is possible that as several recent reports have shown, the NH2 terminus of AML1...
(RUNX1), which is maintained in AME, is necessary together with GATA1 for expression of several megakaryocytic markers and for progression of megakaryopoiesis (see refs. 49, 50 for recent reviews). These reports together with previous finding from our group and others that this fusion protein is also capable of blocking the transforming growth factor \( \beta \) pathway that controls megakaryocyte expansion in the BM (51–53) could help to understand the dominant role that AME plays in alteration of the megakaryocytic program. In any event, these data suggest that in a large proportion of ET patients there is deregulation of STAT3 that affects preferentially the megakaryocytic program raising the possibility that hyperactivation of STAT3 could be one of the primary defects in some ET patients. Our novel model should provide the tools to dissect the pathways that are involved.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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