Effects of Chromatin-Modifying Agents on CD34+ Cells from Patients with Idiopathic Myelofibrosis

Jun Shi, Yan Zhao, Takefumi Ishii, Wenyang Hu, Selcuk Sozer, Wei Zhang, Edward Bruno, Valerie Lindgren, Mingjiang Xu, and Ronald Hoffman

Abstract
Idiopathic myelofibrosis (IM) is likely the consequence of both the acquisition of genetic mutations and epigenetic changes that silence critical genes that control cell proliferation, differentiation, and apoptosis. We have explored the effects of the sequential treatment with the DNA methyltransferase inhibitor, decitabine [5-aza-2’-deoxycytidine (5azaD)], followed by the histone deacetylase inhibitor, trichostatin A (TSA), on the behavior of IM CD34+ cells. Unlike normal CD34+ cells where 5azaD/TSA treatment leads to the expansion of CD34+ cells and narrow-repopulating cells, treatment of IM CD34+ cells results in a reduction of the number of total cells, CD34+ cells, and assayable hematopoietic progenitor cells (HPC). In IM, HPCs are either heterozygous or homozygous for the JAK2V617F mutation or possess wild-type JAK2 in varying proportions. Exposure of IM CD34+ cells to 5azaD/TSA resulted in a reduction of the proportion of JAK2V617F-positive HPCs in 83% of the patients studied and the reduction in the proportion of homozygous HPCs in 50% of the patients. 5azaD/TSA treatment led to a dramatic reduction in the number of HPCs that contained chromosomal abnormalities in two JAK2V617F-negative IM patients. IM is characterized by constitutive mobilization of HPCs, which has been partly attributed to decreased expression of the chemokine receptor CXCR4. Treatment of IM CD34+ cells with 5azaD/TSA resulted in the up-regulation of CXCR4 expression by CD34+ cells and restoration of their migration in response to SDF-1. These data provide a rationale for sequential therapy with chromatin-modifying agents for patients with IM. [Cancer Res 2007;67(13):6417–24]

Introduction
Idiopathic myelofibrosis (IM) is a chronic myeloproliferative disorder (MPD) characterized by cytopenias, a leukoerythroblastic blood picture, splenomegaly, teardrop RBC, marrow fibrosis, constitutive mobilization of CD34+ cells, extramedullary hematopoiesis, and increased bone marrow (BM) microvessel density (1). In approximately half of patients with IM, hematopoietic cells possess either the JAK2V617F or MPLW515L/K somatic mutations which are activating mutations that confer increased sensitivity to hematopoietic cytokines (2–7). JAK2V617F is a subtle mutation that induces a limited gain of function in JAK2. There is evidence that its activation requires the presence of cytokine receptors to induce signaling (8). The number of V617F JAK2 copies likely contributes to the phenotypic heterogeneity of the JAK2V617F myeloproliferative disorders. About one-third of patients with polycythemia vera (PV) have a biallele (homozygous) JAK2V617F mutation accompanied by a 9p loss of heterozygosity, whereas the remainders have a single mutational allele (monoallele). The frequency of a biallelic homozygous JAK2V617F is significantly higher in patients with myelofibrosis developed with a preceding history of PV than in essential thrombocythemia-related myelofibrosis or patients with primary IM (9).

IM is characterized by the constitutive mobilization of primitive hematopoietic progenitor cells (HPC) and hematopoietic stem cells (HSC; refs. 10–13). Barosi et al. (10) recently showed that the peripheral blood (PB) of IM patients contained 360 times more CD34+ cells than normal individuals and 18 to 30 times more CD34+ cells than patients with other Philadelphia chromosome-negative (Ph−) MPDs. In addition, the PB CD34+ cell number was further shown to be related to disease progression and to serve as a biomarker for disease activity in patients with IM (10). The number of assayable HPCs presented in the PB of IM patients has also been shown to be increased (10–13). Furthermore, we have reported that PB CD34+ cells in IM contain not only HPCs but also BM-repopulating cells belonging to the malignant clone (13). Our laboratory has shown that IM is characterized by a proteolytic environment that contributes to the sustained mobilization of CD34+ cells (13, 14). The increased trafficking of CD34+ cells in IM, therefore, seems to be a distinctive biological characteristic of IM.

In normal individuals, only a small fraction of HSCs/HPCs regularly exit the BM and circulate in the PB (15). Maintenance of the balance between mobilization, homing, and retention of HSCs/HPCs within the marrow microenvironment is an important characteristic of normal adult BM homeostasis. Stem cell trafficking is largely dependent on the interaction between a number of integrins and chemokine receptors expressed by stem cells and progenitors with a variety of matrix proteins and chemokines elaborated by marrow fibroblasts, osteoblasts, or endothelial cells (16). The CXC chemokine Stromal-derived factor-1 (SDF-1) plays a major role in migration, proliferation, differentiation, and survival of many cell types, including human hematopoietic stem/progenitor cells. CXC-chemokine receptor-4 (CXCR4), the 7-transmembrane receptor of SDF-1 is widely expressed by a variety of hematopoietic cell lineages (16). SDF-1/CXCR4 interactions are thought to play an important role in stem cell migration and differentiation (16, 17). We have previously reported that CXCR4 expression was down-regulated in the CD34+ cells of patients with IM; this abnormality was
thought to contribute to the constitutive mobilization of hematopoietic stem and progenitor cells that occurs in patients with IM (14, 18).

The malignant phenotype in IM likely results from a combination of genetic abnormalities and epigenetic modifications leading to the dysregulation of critical genes that contribute to cell proliferation, differentiation, and cell death (2–7, 19–21). Methylation of cytosine residues in the promoter region as well as transcriptional inhibitory complexes including histone deacetylases (HDAC) play a role in the transcriptional silencing of genes in a variety of human cancers (22–24). A considerable amount of data has been reported which indicates that re-expression of most silenced genes through promoter demethylation requires sequential application of DNA methyltransferase (DNMT) inhibitors followed by HDAC inhibitors (22–30).

In this report, we have explored the sequential use of 5-aza-2'-deoxycytidine (5azaD) and trichostatin A (TSA) for the treatment of IM. We determined that treatment of IM CD34+ cells with chromatin-modifying agents resulted in the up-regulation of functional CXCR4 expression by CD34+ cells and to the reduction of the number of JAK2V617F-positive progenitor cells. It is anticipated that such data might serve as a potential platform with which to proceed with clinical trials of these agents in patients with IM.

Materials and Methods

Patients. PB was collected from 16 patients with IM after informed consent was obtained according to the guidelines of the Institutional Review Board at the University of Illinois College of Medicine. All patients met the WHO diagnostic criteria for IM (31). None of the patients were receiving cytotoxic agents at the time of study.

Purification of IM PB CD34+ cells. The PB was layered onto Ficoll- Hypaque (1.077 g/mL; Amersham Biosciences) and low-density mononuclear cells separated after centrifugation. Granulocytes were isolated by standard techniques as previously described (4–6). A CD34+ cell population was isolated using a human CD34+ cell selection kit (StemCell Technologies) according to the manufacturer's instructions. The purity of the CD34+ cell population was analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Cell fractions having a CD34+ cell purity of 90% or greater were used for all experiments.

Ex vivo cultures exposed to 5azaD and TSA. An ex vivo culture system for IM CD34+ cells was established as previously described (32–34). Briefly, IM PB CD34+ cells (1 × 10^6 per well) were cultured in 2.5 mL Iscove's modified Dulbecco's medium (IMDM; Bio Whittaker) containing 30% fetal bovine serum (FBS; HyClone Laboratories) supplemented with 100 ng/mL stem cell factor (SCF), 100 ng/mL FLT-3 ligand (FL), 100 ng/mL thrombopoietin, and 50 ng/mL interleukin-3 (IL-3; Amgen) and incubated in a humidified incubator maintained at 37 °C with 5% CO2. After an initial 16 h of incubation, cells were exposed to 5azaD (Pharmachemie B.V.) at a concentration of 1 × 10^6 mol/L. After 48 h, cells were washed and then distributed to new culture plates containing SCF, FL, and thrombopoietin with TSA (Sigma), which was added once at the beginning of the following 7-day culture done at a concentration 16.5 nmol/L. In addition, identical cultures to which 5azaD/TSA were not added were done in parallel. In some experiments, the CD34+ cells were reisolated after 9 days of culture and assayed for their ability to migrate in response to SDF-1 or to form hematopoietic colonies.

Flow-cytometric analysis. After 9 days of culture, cells were stained with anti-human CD34 monoclonal antibody (mAb) conjugated to FITC in combination with anti-human CXCR4 (CD184) or CD90 conjugated to phycoerythrin. All mAbs were purchased from Becton Dickinson PharMin- gen. Cells were incubated with specific mAb or isotype-identical control immunoglobulin G for 30 min on ice in the dark. Immediately before fluorescence-activated cell sorting analysis, 1 µg/mL propidium iodide (Sigma) was added for the exclusion of nonviable cells. Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson), and at least 10,000 live cells were acquired for each analysis (Cell-Quest software; Becton Dickinson). Both the mean fluorescence intensity (MFI) of CXCR4 expression on CD34+ cells and the percentage of CXCR4+CD34+ cells within the CD34+ cell population were assessed.

Hematopoietic progenitor cell assays. Primary IM CD34+ cells or IM CD34+ cells reisolated following culture with cytokines alone or cytokines plus chromatin-modifying agents were assayed in semisolid media as described previously (14, 33). Briefly, 5 × 10^3 cells were plated per dish in duplicate cultures containing 1 mL IMDM with 1.1% methylcellulose, 30% FBS, 5 × 10^−5 mol/L 2-mercaptoethanol (Cell Technologies), to which 100 ng/mL SCF, 100 ng/mL IL-3, 100 ng/mL IL-6, 100 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF), and 5 units/mL erythropoietin were added. Colonies were enumerated after 14 days of incubation, and individual colonies were plucked and genotyped for the JAK2V617F mutation. The percentage of JAK2V617F-positive colonies formed by CD34+ cells before and following 9 days of culture was determined. In two cases of JAK2V617F-negative IM, cytokin preparations (Shandon) of plucked colonies were prepared, and the percentage of cells belonging to the malignant clone was determined by interphase fluorescence in situ hybridization (FISH) for a marker chromosomal abnormality.

Migration assay. The migration behavior of primary IM CD34+ cells before and following cell culture was done as previously described using 6.5-mm-diameter, 5-µm-pore transwell plates (Corning). Briefly, transwell filters were coated overnight at 4 °C with 10 mg/cm² of fibronectin (Sigma) at a concentration of 20 µg/mL in PBS. The coating solution was aspirated and replaced by a 1% bovine serum albumin (BSA) solution in PBS at 37 °C for 30 min to block nonspecific binding sites. Before cells were added to the upper compartment, the coated transwell filters were washed twice with transwell buffer (IMDM with 0.5% BSA). Then, 100,000 to 200,000 CD34+ cells in 100 µL transwell buffer were placed in the upper chamber of the transwell. About 600 µL transwell buffers containing 200 ng/mL SD-1 were added to the lower compartment. After incubation at 37 °C for 4 h, nonmigrating and migrating cells were harvested from the upper and lower compartments, respectively. Nonmigrating cells were recovered following two washes each consisting of a 5-min treatment with an enzyme-free cell dissociation buffer (Life Technologies) at 37 °C, followed by vigorous pipetting. The harvested cells in the two fractions were enumerated using a hemacytometer. The percentage of cells migrating was calculated by determining the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment.

Quantitative real-time reverse transcription-PCR assay of CXCR4 mRNA and the percent of JAK2V617F/JAK2wt. Total RNA was extracted from primary IM CD34+ and CD34− cells (50,000–100,000) reisolated after culture by using a PicoPure RNA Isolation Kit (Arcturus Bioscience). First-strand complimentary DNA (cDNA) was synthesized from total RNA with SuperScriptTM III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The sequences of the primers used for the amplification were as follows: CXCR4 (161 bp), 5′-AGGAGGATCAGTTATATCACCTT-3′ (forward), 5′-TGCCCAAATGCGCAATTTAG-3′ (reverse); (β-actin (176 bp), 5′-AGCCCTGCCTTTGCGCA-3′ (forward), 5′-CTGTGTTGCCGT-GGGGG-3′ (reverse). The quantitative real-time reverse transcription-PCR (RT-PCR) assay were done with the ABI Prism 7900 Sequence Detection System (Applied Biosystems), and the PCR products were detected by the use of SYBR green technology (ABI). First-strand complimentary DNA (cDNA) was synthesized from total RNA with SuperScriptTM III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The sequences of the primers used for the amplification were as follows: CXCR4 (161 bp), 5′-AGGAGGATCAGTTATATCACCTT-3′ (forward), 5′-TGCCCAAATGCGCAATTTAG-3′ (reverse); (β-actin (176 bp), 5′-AGCCCTGCCTTTGCGCA-3′ (forward), 5′-CTGTGTTGCCGT-GGGGG-3′ (reverse). The quantitative real-time reverse transcription-PCR (RT-PCR) assay were done with the ABI Prism 7900 Sequence Detection System (Applied Biosystems), and the PCR products were detected by the use of SYBR green technology (ABI). Cycling conditions included initial denaturation at 95 °C for 10 min, then by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. All assays were done in triplicate, and a negative control was included in each assay. To quantify CXCR4 mRNA expression in relation to an internal control, an amplification standard curve of the reference gene β-actin was established as previously described (34, 36). The CXCR4 mRNA expression was quantified by measuring threshold cycle and presented as relative rates compared with the expression of the reference gene β-actin.
Genomic DNA (gDNA) was extracted from granulocytes using the Easy-DNA Kit (Invitrogen) and was used for the determination of the percent of JAK2V617F/JAK2total by real-time quantitative kinetic PCR assay using the allelic discrimination method as previously used in our laboratory (37).

Nested allele-specific PCR for JAK2V617F-positive colonies. Individual hematopoietic colonies were randomly plucked from the semisolid media, and gDNA was isolated by using the Extract-N-Amp Blood PCR Kits (Sigma). The JAK2V617F mutation was detected by using nested allele-specific PCR as previously described (38). Briefly, a 521-bp DNA fragment containing the V617F mutation site was amplified from the gDNA using primers P1 5‘-GATCTCCTATCGCAGCTTACA-3’ and P1r 5‘-TATGGTT-TTGGGCATTTGAACTCCT-3’. After 35 cycles consisting of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, 0.5-μL PCR products were further amplified by nested and allele-specific primers P2 5‘-CCCTAGAGCCTGATTGCGA-3’, P2r 5‘-ATTGCTTTCCTTCTTCAAGA-3’, Pnd 5‘-AGCATTTGGTTTATATATG-3’, and Pmr 5‘-GGTTTACTAATCCTCGTCCAAACAA-3’, following 35 cycles of 95°C for 30 s, 59°C for 25 s, and 72°C for 25 s. The final PCR products were analyzed on 2.5% agarose gels. The nested PCR product had a size of 453 bp. A 279-bp product indicated allele-specific JAK2V617F positive whereas a 229-bp product denoted allele-specific wild product. Colonies classified as homozygous for JAK2V617F contained only a 279-bp band, whereas heterozygous colonies were identified based on the presence of both the 279-bp and 229-bp bands.

Detection of isochromosome 17 and monosomy 7 by interphase FISH. CD34+ cells from two patients with known cytogenetic abnormalities, isochromosome 17q and monosomy 7, respectively, were studied in an identical fashion. Cytosin preparations of pooled plated hematopoietic colonies assayed following exposure to cytokines or cytokines plus chromatin-modifying agents were made. Interphase FISH was done using probes for isochromosome 17 (RARA in 17q11.2) and monosomy 7 (D7Z1 at the centromere of chromosome 7) according to the manufacturer’s (Abbott Molecular) instructions (13). Three signals, rather than the normal two, from the RARA gene probe were indicative of an isochromosome 17q and one signal for D7Z1, instead of the normal two, indicated monosomy of chromosome 7. In each experiment, 100 cells were scored.

Statistical analysis. Results are reported as mean ± SD of data obtained from varying numbers of individual experiments. Statistical significance was determined using Student’s t tests or paired-samples t test. All P values are two-sided.

Results

CD34+ cells phenotype after 5azaD/TSA treatment. IM CD34+ cells (1 × 10^6) generated greater numbers of cells in the presence of cytokines alone (16.7 ± 14.3 × 10^5) than in the presence of cytokines plus 5azaD/TSA (6.7 ± 4.6 × 10^5; P < 0.05). IM CD34+ cells were phenotypically characterized before and following 9 days of exposure to cytokines alone or cytokines plus 5azaD/TSA. The proportion of CD34+ cells in cultures containing 5azaD/TSA (11.3 ± 8.9%) was greater than in cultures containing cytokines alone (6.3 ± 7.3%). Furthermore, as can be seen in Fig. 1A, 68.7 ± 17.6% of the CD34+ cells after 9 days of culture in the presence of 5azaD/TSA coexpressed CD34+ and CD90+, whereas only 36.3 ± 18.1% of the cells in the cultures exposed to cytokines alone were CD34+CD90+ (P < 0.001). The absolute number of IM CD34+ cells and CD34+CD90+ cells in both cultures expressed to cytokines alone or cytokines plus 5azaD/TSA represented almost half of the number present in the input population (data not shown).

As shown in Fig. 2A, the percentage of cells within the 5azaD/TSA-treated IM CD34+ cells fraction which expressed CXCR4 was significantly greater (47.8 ± 15.4%; P = 0.006) than that of the cells exposed to cytokines alone (30.3 ± 13.9%) or primary IM CD34+ cells (12.8 ± 6.4%). In addition, the absolute number of CD34+ cells expressing CXCR4 was 2- to 10-fold greater following treatment with 5azaD/TSA as compared with the input number of CD34+CXCR4+ cells (data not shown). These data show that the proportion of CD34+ cells that are CXCR4+ increases after 9 days of culture in the presence of cytokines alone, and that this proportion is further enhanced in cells exposed to cytokines plus 5azaD/TSA. To determine whether the level of CXCR4 receptor expression increased on 5azaD/TSA-treated CD34+ cells, we analyzed the CXCR4 MFI on the CD34+ cells. As shown in Fig. 2B, CXCR4 MFI of the 5azaD/TSA-treated CD34+ cells was 47.0 ± 29.5, which was significantly greater (P = 0.021) than that of CD34+ cells exposed to cytokines alone (24.6 ± 13.8) or primary IM CD34+ cells (11.5 ± 3.8). These results strongly indicate that 5azaD/TSA treatment leads to the up-regulation of CXCR4 receptor expression by IM PB CD34+ cells.

Effect of 5azaD/TSA treatment on CXCR4 mRNA expression. We have previously reported that CXCR4 mRNA expression was reduced in IM PB CD34+ cells and granulocytes as compared with their normal counterparts (14, 18). We tested if CXCR4 transcripts were up-regulated following exposure of IM PB CD34+ cells to 5azaD/TSA. CXCR4 mRNA expression level in CD34+ cells was normalized to β-actin and was assessed by quantitative real-time RT-PCR. As shown in Fig. 2C, relatively higher level of CXCR4 transcripts (2.95 ± 1.65) were observed in CD34+ cells treated with 5azaD/TSA as compared with the cells in cultures exposed to cytokines alone (1.07 ± 0.63, P = 0.005).

Migration capacity of IM CD34+ cells exposed to chromatin-modifying agents. To assess the functional consequences of the up-regulation of CXCR4 expression by IM CD34+ cells, we examined the migration of 5azaD/TSA-treated CD34+ cells in response to SDF-1 in vitro in comparison with CD34+ cells reisolated from cultures supplemented with cytokines alone as well as primary CD34+ cells. IM PB CD34+ cells exposed to cytokines alone or cytokines plus 5azaD/TSA were reisolated after 9 days of culture. CD34+ cells were placed in the top chamber of a transwell, and their migration in response to SDF-1 in the bottom chamber was monitored. We determined that 5azaD/TSA-treated CD34+ cells had a greater migration capacity (25.7 ± 7.2%, P = 0.009) toward SDF-1 (Fig. 2D), as compared with CD34+ cells exposed to cytokines only (13.7 ± 5.7%) or primary IM CD34+ cells (5.3 ± 1.7%).

The effects of 5azaD/TSA on the number of assayable HPCs. We have previously reported that 5azaD/TSA treatment resulted in the expansion of the numbers of normal marrow and cord blood progenitor cells (32–34). By contrast, similar 5azaD/TSA treatment of IM CD34+ cells resulted in a marked reduction of all classes of progenitor cells assayable as compared with primary IM CD34+ cells and IM CD34+ cells exposed to cytokines alone (Fig. 3).

5azaD/TSA treatment reduces the burden of JAK2V617F-positive colonies. The JAK2V617F allele status of granulocytes isolated from the 16 patients with IM was determined. A total of 10 of the 16 patients were JAK2V617F positive, and in 6 of 10 patients, the effects of treatment with 5azaD/TSA on the numbers of JAK2V617F-positive colonies were assessed. As can be seen in Table 1, three of the six patients were heterozygous, and three were homozygous for the mutant allele.

To assess the effect of 5azaD/TSA treatment on the burden of malignant progenitor cells, CD34+ cells from these IM patients who harbored the JAK2V617F mutation were studied. CD34+ cells were cultured for 9 days with cytokines alone or cytokines plus 5azaD/TSA, and the CD34+ cells were reisolated. Primary and reisolated CD34+ cells were plated in semisolid media, and half of the total colonies were randomly plucked after 14 days of culture and analyzed for JAK2V617F mutation using nested allele-specific PCR.

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As can be seen in Table 1, 34% to 100% of the hematopoietic colonies cloned from primary IM CD34+ cells were JAK2V617F positive, and 40% to 100% of these colonies were homozygous for the mutation, whereas the remainder of the JAK2V617F-positive colonies was heterozygous or wild type. In patients IM3, IM10, IM31, and IM35, the presence of colonies that contained wild-type JAK2 indicates that there is a population of normal progenitors that persists in some IM patients. All of the colonies derived from primary IM CD34+ cells of IM12 and IM17 were JAK2V617F positive, and all of the colonies assayed from IM 12 CD34+ cells were homozygous for the mutation, whereas in IM17, 62% of the colonies were homozygous, and 38% were heterozygous for JAK2V617F. These data indicate that in JAK2V617F-positive IM, wild-type, monoallelic, and biallelic progenitors are present in varying proportions. Exposure of IM CD34+ cells to cytokines alone for 9 days did not result in a reduction of the numbers of JAK2V617F-positive colonies or the numbers of colonies that were homozygous. By contrast, treatment of IM CD34+ cells with cytokines plus 5azaD/TSA resulted in a significant reduction of the proportion of colonies that were JAK2V617F positive (Fig. 4), as compared with cytokines alone (53 ± 34% versus 80 ± 15%, P = 0.035, paired-samples t-test). We also observed a reduction in the proportion of JAK2V617F homozygous colonies following the exposure to 5azaD/TSA as compared with cytokines alone in three of six patients studied.

5azaD/TSA treatment also reduces the proportion of progenitor cells that harbor cytogenetic abnormalities. We studied two patients with JAK2V617F-negative IM that were characterized by chromosomal abnormalities. The percentage of malignant cells within the pooled colonies following treatment with cytokine alone or cytokine plus 5azaD/TSA was monitored by FISH analysis (Table 2). In both instances, 5azaD/TSA resulted in a dramatic reduction of the numbers of colonies that contained cells with the chromosome abnormality. None of the JAK2V617F-positive patients studied had marker chromosome abnormalities.

Discussion

The initiation and progression of human malignancies are thought to be due not only to the acquisition of genetic mutations but also to epigenetic changes that are not caused by alterations in the primary nucleotide sequence of DNA (22–30). These two processes are closely interrelated in promoting the development of cancer. Recently, several somatic mutations in either a tyrosine kinase (JAK2) or a cytokine receptor (mpl) have been reported in a large number of patients with MPDs, including IM (2–7). A substantial amount of evidence has been generated which indicates that these somatic mutations play a significant role in the pathobiology of the MPDs. In addition to these genetic mechanisms, a growing number of investigators have provided data which indicate that epigenetic mechanisms silence genes that impede cellular proliferation or movement in IM (19–21). Rossi et al. (39) have reported aberrant methylation of a variety of negative regulators of JAK2 activation/phosphorylation, including SHP-1,
SOCS-1, and SOCS-3, which have been observed in patients with both JAK2V617F-positive or JAK2V617F-negative IM. Furthermore, Opalinska et al. did high-resolution epigenomic mapping of IM cells, and revealed high levels of functionality important methylation. This high rate of methylation in IM suggests that epigenetic silencing of genes may play an important role in the pathogenesis of this disorder (40).

Other modifications of the chromatin structure have been associated with gene silencing. For instance, removal of acetyl groups from histone lysine tails is associated with transcriptional

Figure 2. A–D, functional expression of CXCR4 by IM CD34⁺ cells following exposure to chromatin-modifying agents. A, after ex vivo culture in the presence of cytokines alone, the proportion of CD34⁺CXCR4⁺ cells increased as compared with primary CD34⁺ cells (n = 9). The addition of 5azaD/TSA led to an even greater increase in the expression of CXCR4 (n = 13; P = 0.006). B, 5azaD/TSA-treated CD34⁺ cell population also had the highest MFI of CXCR4 as compared with primary CD34⁺ cells (n = 9) or CD34⁺ cells exposed to cytokines alone (n = 13; P = 0.021). C, up-regulation of CXCR4 mRNA expression occurred in the 5azaD/TSA-treated CD34⁺ cells (n = 8). Total RNA was extracted from reisolated CD34⁺ cells after 9 d of culture in the presence of cytokines alone or cytokines plus 5azaD/TSA treatment. The relative expression levels CXCR4 mRNA was determined by real-time quantitative PCR. β-Actin was used as internal calibrator (control gene). A 3.16 ± 1.14-fold increase in CXCR4 mRNA expression after 5azaD/TSA treatment (n = 8, paired-samples t test; P = 0.005) was observed as compared with cultures containing cytokines alone. D, 5azaD/TSA-treated CD34⁺ cells had a greater migratory potential in response to SDF-1. CD34⁺ cells cultured in the presence of cytokines plus 5azaD/TSA showed significantly higher migration ability as compared with CD34⁺ cells reisolated from cultures containing cytokines alone (n = 6; P = 0.01) or primary CD34⁺ cells (n = 4).
silencing (22–30). In cultures of cancer cells, the administration of HDAC inhibitors will not result frequently in the re-expression of densely hypermethylated genes unless DNMT inhibitors are first given; the two inhibitors are then synergistic for re-expression of genes (27). In myeloid malignancies, the use of chromatin-modifying agents that target epigenetically silenced transcription may activate a genetic progress, leading to cellular differentiation, apoptosis, or senescence, allowing normal stem cells to replace the molecular stem cell populations (27, 30). The sequential application of HDAC inhibitors following DNMT inhibitors have been proposed to potentially increase the response rate, response duration, or percentage of complete remissions in myeloid leukemia by promoting the re-expression of important silenced regulatory genes that might have a profound antitumor effect or lead to the induction of cellular differentiation (30). Because effective therapeutic options for the treatment of patients with IM are few, we have explored the potential role of chromatin-modifying agents in the treatment of IM using an in vitro culture system.

Our laboratory has previously reported that the sequential use of chromatin-modifying agents has a profound effect on normal HSCs, resulting in their ability to undergo symmetrical cell division with retention of their marrow-repopulating potential (32–34). Unlike malignant cells, the addition of sequential 5azaD/TSA in the presence of a cytokine cocktail favors the division of normal primitive HPCs which retain their marrow-repopulating potential. This behavior is in contrast to stem cells exposed in vitro to cytokines alone which undergo progressive loss of their proliferative and self-renewal capacity. Sequential treatment of normal stem cells with 5azaD/TSA results in the up-regulation of expression of number of genes previously implicated in HSCs self-renewal (34). Reversal of the sequence of the administration of these two agents has, however, resulted in the loss of their effects on the behavior of normal CD34+ cells (33). The effects of chromatin-modifying agents on myeloid neoplasias could, therefore, be due not only to their causing cytotoxicity and terminal differentiation of malignant cells, but also preferential expansion of the pool of residual normal stem cells.

In the present report, we showed that the in vitro administration of sequential 5azaD/TSA had a profound effect on IM CD34+ cells. The effects of these chromatin-modifying agents were different from that observed when normal CD34+ cells were treated in an identical manner. 5azaD/TSA resulted not only in a dramatic reduction in the number of assayable IM progenitor cells, but also in the numbers of CD34+ cells and CD34+CD90+ cells. The burden of progenitor cells belonging to the malignant clone in IM was

### Table 1. Effect of 5azaD/TSA treatment on CD34+ cells with JAK2V617F-positive IM

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<th>Granulocyte*</th>
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<th>Treatment 2</th>
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<tr>
<td></td>
<td>Cytokines alone</td>
<td>Cytokines + 5azaD/TSA</td>
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<tr>
<td>JAK2V617F(%)</td>
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<td>60</td>
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<tr>
<td>IM35</td>
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<td>34 (23/68)</td>
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*Indicates the percentage of JAK2V617F allele obtained from the real-time PCR analysis of IM PB granulocytes.

1 Each value represents the percentage of colonies exhibiting the JAK2V617F mutation or homozygous JAK2V617F allele.

2 Indicates colonies assayed from primary CD34+ cells. The numbers in parentheses denote the actual number of JAK2V617F-positive colonies/the total numbers of colonies analyzed.

3 There was a significant reduction of the percentage of JAK2V617F-positive colonies derived from IM CD34+ cell culture in the presence of 5azaD/TSA as compared with colonies assayed from CD34+ cells exposed to cytokines alone (53 ± 34% versus 80 ± 15%, P = 0.035, paired-samples t test).
tracked using quantitative PCR of JAK2V617F alleles in patients with JAK2V617F mutation or by monitoring the persistence of cytogenetic abnormalities using FISH analysis of hematopoietic colonies cloned from CD34+ cells isolated from patients with JAK2V617F-negative IM. Using both strategies, we showed that sequential 5azaD/TSA treatment resulted in a reduction in the numbers of malignant progenitor cells. The data generated by monitoring the number of assayable progenitors that were JAK2V617F positive were especially revealing. In five of the six patients studied, primary CD34+ cells produced not only in vitro hematopoietic colonies that were homozygous for JAK2V617F, but also a fraction that contained cells that were heterozygous for JAK2V617F or contained wild-type JAK2. By contrast, in another patient (IM12), all of the hematopoietic colonies were JAK2V617F homozygous. These data indicate that JAK2V617F-positive IM resembles PV in that JAK2V617F homozygous clones may predominate with time. The treatment of IM CD34+ cells with cytokines alone did not substantially change the percentage of JAK2V617F colonies or the numbers of homozygous colonies. By contrast, treatment with cytokines and sequential 5azaD/TSA resulted in a reduction of the percentage of JAK2V617F-positive colonies in five of six patients and the appearance of greater numbers of colonies with wild-type JAK2 which likely represents the progeny of a normal reservoir of progenitor cells. Furthermore, in three of the six patients, the treatment of IM CD34+ cells with chromatin-modifying agents resulted in the reduction of the proportion of JAK2V617F homozygous colonies. This preferential suppression of the numbers of JAK2V617F homozygous colonies by treatment with chromatin-modifying agents could be due to either their preferential elimination by a direct cytotoxic effect of the drug or the increased expression of previously suppressed genes that results in the transcription of gene products that leads to cell death or loss of their proliferative advantage as compared with JAK2V617F heterozygous progenitor cells or wild-type progenitor cells.

The constitutive mobilization of CD34+ cells is a defining characteristic of IM, which results ultimately to the development of extramedullary hematopoiesis (1, 10). Our laboratory has previously reported that IM CD34+ cells are characterized by the reduced expression of the chemokine receptor, CXCR4 (14, 18). Because CXCR4/SDF-1 interactions are thought to play a role in normal stem cell trafficking, the reduction of IM CD34+ CXCR4 expression has been hypothesized to contribute to this abnormal stem cell trafficking. Rosti et al. have shown that transcriptional defects are the major cause of the CXCR4 down-regulation in IM (18). CXCR4 down-regulation on IM CD34+ cells is associated with advanced patient age, the presence of severe anemia, thrombocytopenia, and degree of marrow fibrosis (18). Treatment of IM CD34+ with cytokines plus 5azaD/TSA for 9 days was associated with an increase in the numbers of CD34+ cells expressing CXCR4 as well as an increased density of CXCR4. In vitro exposure of normal stem/progenitor cells to cytokines has been previously reported to up-regulate both cell surface and intracellular CXCR4, restoring their migratory behavior toward SDF-1 (41). 5azaD/TSA treatment resulted in a far more dramatic (5-fold) increase in CXCR4 expression by IM CD34+ cells than was

### Table 2. Effect of 5azaD/TSA on CD34+ cells with JAK2V617F-negative IM

<table>
<thead>
<tr>
<th></th>
<th>Percentage of cells within hematopoietic colonies with marker chromosome abnormality</th>
<th>Percentage of reduction of cells with marker chromosome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines alone</td>
<td>Cytokines + 5azaD/TSA</td>
<td></td>
</tr>
<tr>
<td>IM2 (iso17)</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>IM13 (mono7)</td>
<td>39</td>
<td>35</td>
</tr>
</tbody>
</table>

NOTE: Each number represents the percentage of 100 cells with a marker chromosome as determined by FISH analysis. The percentage of reduction of cells with the marker chromosome abnormality was determined by dividing (the percentage of cells with the marker chromosome in cultures exposed to cytokines alone – the percentage of cells with the marker chromosome in cultures exposed to cytokines plus drug treatment) by the percentage of cells with abnormal chromosome marker with cytokines alone.

Abbreviations: iso17, isochromosome 17; mono7, monosomy 7.
associated with the expression of IM CD34+ cells to cytokines. This increase in IM CD34+ cells CXCR4 expression was also associated with increased migration capacity of drug-treated CD34+ cells toward SDF-1. This increased expression of functional CXCR4 by IM CD34+ cells was attributed to increased transcription of CXCR4 in IM CD34+ cells treated with chromatin-modifying agents. Epigenetic regulation of CXCR4 in human tumors has been previously recognized (42–44). Furthermore, constitutive as well as inducible expression of CXCR4 has been reported to be restored following the treatment of both pancreatic cancer cells as well as human melanoma cells by chromatin-modifying agents (42, 43). The up-regulation of CXCR4 expression by IM CD34+ cells following treatment with 5azaD/TSA treatment shows an important epigenetic mechanism that may endogenously regulate chemokine receptor expression in IM CD34+ cells.

The findings in this report, therefore, provide a rationale for the use of chromatin-modifying agents to treat patients with IM in a manner that may potentially alter its natural history. A clinical trial to determine if the sequential administration of a DNMT inhibitor followed by a HDAC inhibitor could be tolerated by IM patients is of great importance. Furthermore, such a trial might determine if the up-regulation of CXCR4 by IM CD34+ cells, as well as the reduction of the numbers of JAK2V617F progenitor cells, could be achieved in patients by the administration of these agents and would alter the natural history of this hematologic malignancy.

Acknowledgments

Received 2/12/2007; revised 4/1/2007; accepted 4/19/2007.

Grant support: National Cancer Institute (1P01CA10671 to R. Hoffman), the Department of Defense (MP08010 to R. Hoffman and MP08007 to M. Xu), and the Myeloproliferative Disorders Foundation (R. Hoffman and M. Xu).

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We thank Melinda H. Hatcher for her excellent technical assistance.

References

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