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Effects of the MEK inhibitor CI-1040 (PD 184352) on progenitor growth from normal and myelodysplastic marrow

Previous studies demonstrated that ~50% of acute myelogenous leukemia samples exhibit activation of the mitogen-activated protein kinase (MAPK) pathway[1-3] and growth inhibition by MEK inhibitors.[3] To extend these observations, we assessed MAPK pathway activation and effects of CI-1040 in normal and myelodysplastic (MDS) marrow.

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Activation of the mitogen-activated protein kinase (MAPK) pathway, as manifested by phosphorylation of extracellular signal regulated kinases (ERK) 1 and 2 on a specific Thr-X-Tyr motif, is usually assessed by immunoblotting.

Because normal and myeloblastic (MDS) marrow contain mixed cell populations, immunohistochemistry was utilized to assess cell type-specific pathway activation. Under Institutional Review Board-approved protocols, B5-fixed, paraffin-embedded marrows from 14 MDS patients (3 RA, 5 RAEB, 3 RCMD, 3 CMML) and 4 controls were treated with 3% H_2O_2 to inhibit peroxidases, subjected to heat-induced antigen retrieval, and stained with rabbit anti-phospho-ERK1/2 (anti-ACTIVE™ p44/42 MAPK; Promega, Madison, WI, USA) followed by biotinylated secondary antibody and horseradish peroxidase-conjugated streptavidin. Fixed, paraffin-embedded HL-60 leukemia cells and normal human tonsil served as positive and negative controls, respectively. This analysis demonstrated MAPK activation in all MDS marrows examined. As illustrated in Figure 1A, staining was strongest in blast cells and myeloid progenitors, intermediate in erythroid cells, and weakest in megakaryocytes. The staining strength and pattern did not differ among MDS subtypes. In normal marrow, a similar pattern was observed, although the staining intensity was lower (Figure 1B).

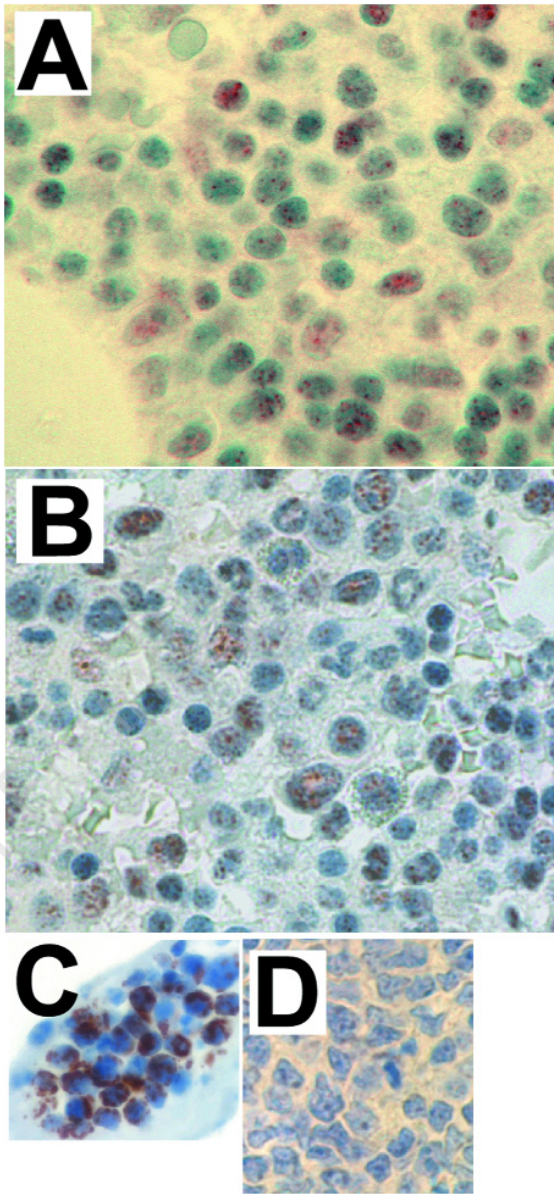

Figure 1. Immunohistochemical staining for dually phosphorylated ERK1/2 in MDS and normal marrow. A, marrow biopsy specimen from a patient with RAEB. B, marrow biopsy specimen from a normal control. C, phospho-ERK staining in HL-60 cells, which are known to have MAPK activation.[3,9] D, lack of phospho-ERK staining in follicles from normal human tonsil. All panels were counterstained with hematoxylin and photographed at 400X magnification.

In view of these results, the effect of the MEK inhibitor CI-1040[4] on growth of erythroid and myeloid progenitors *in vitro* was examined. Fresh marrow from 12 MDS patients, 14 patients with cytopenias but no evidence of MDS, and 3 normal patients undergoing hip arthroplasty, as well as blood from 5 normal volunteers was fractionated on Ficoll-Hypaque gradients. Mononuclear cells (6×10^5/aliquot) were plated in 3 mL Methocult™ methylcellulose medium (StemCell Technologies; Vancouver, BC, USA) containing diluent or 10 μmol/L CI-

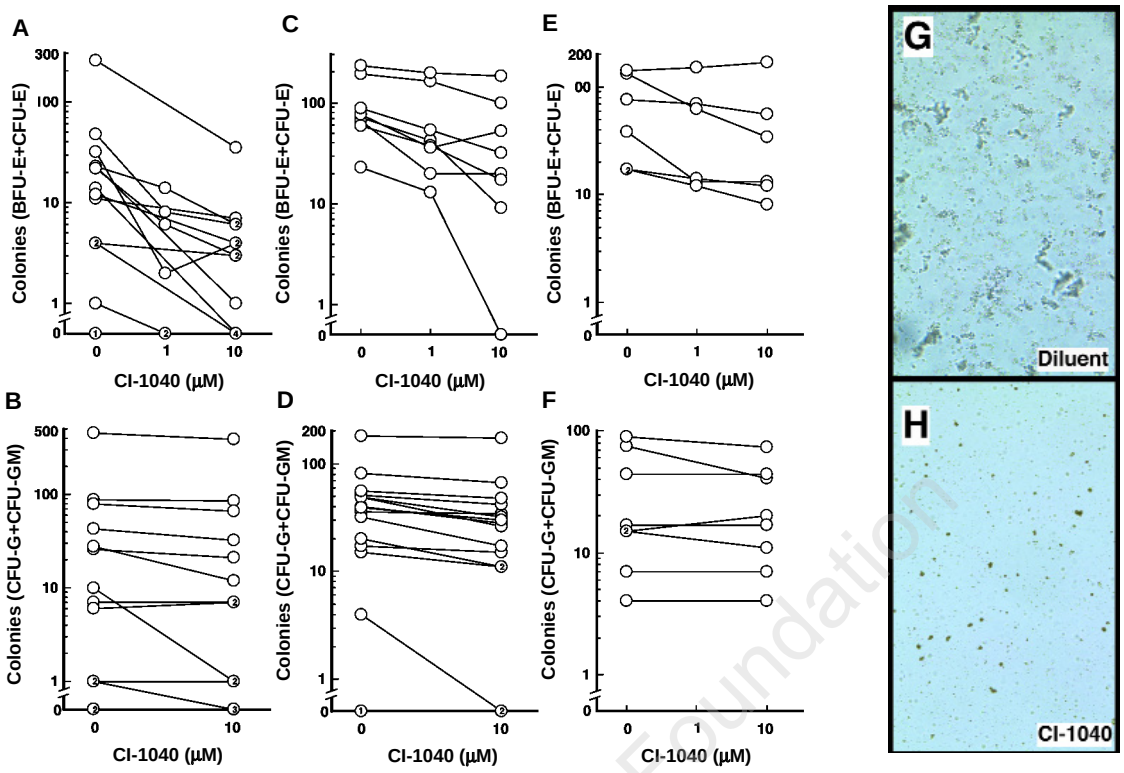

Figure 2. Effects of CI-1040 on erythroid, myeloid and blast colony formation. A-F, erythroid colonies (summed CFU-E and BFU-E; panels A, C and E) or myeloid colonies (summed CFU-G and CFU-GM; panels B, D and F) were enumerated 14 days after samples from patients with MDS (A, B), patients with cytopenias but no evidence of MDS (C, D) or normal marrow (E, F) were plated with the indicated concentration of CI-1040 in 0.1% (v/v) DMSO or the equivalent amount of diluent alone. Numbers in circles, number of different samples with identical colony counts. G, H, blast background (small poorly formed colonies) observed after treatment of the same marrow with 0.1% DMSO (G) or 10 μmol/L CI-1040 (H) in 0.1% DMSO.

1040 (Pfizer, Ann Arbor, MI, USA), a concentration that approximates serum levels of CI-1040 and its active metabolite observed in clinical trials.[5] As specified by the manufacturer of the medium, progenitor colonies (summed BFU-E/CFU-E and CFU-GM/CFU-G) were quantified on day 14 using established morphologic criteria.[6] CFU-GEMM colonies were very scarce in all samples and were not enumerated. Results obtained in these assays are summarized in Figure 2. CI-1040 inhibited erythroid colony formation by >50% in 10 of 12 MDS samples (median inhibition 87%), 5 of 8 non-MDS cytopenic samples (median 67%) and 3 of 6 normal samples (median 41%). Inhibition was greater with 10 μmol/L CI-1040 than with 1 μmol/L, as expected for a dose-dependent process. The degree of inhibition did not vary with MDS subtype. In addition, as illustrated in Figure 2G and H, CI-1040 inhibited by >80% the extensive *blast background* [small clusters of blast-like cells that do not mature or form normal colonies in Methocult™ assays][6] that was present in 3 diluent-treated MDS samples. In contrast, CI-1040 inhibited myeloid colony formation by >50% in only 3 of 11 MDS samples (median inhibition 15%), 1 of 14 non-MDS cytopenic samples (median 25%), and 0 of 8 normal samples (median 0%).

Although a recent biochemical study demonstrated MAPK pathway activation in erythropoietin-treated erythroid progenitors from MDS marrow,[7] to our knowledge this is the first demonstration of MAPK pathway activation in unstimulated normal and MDS marrow. In the absence of exogenous stimuli, erythroid progenitors consistently stained less intensely than myeloid cells (including undifferentiated blasts). While this might reflect lower levels of MAPK activation by endogenous erythropoietic vs. myelopoietic cytokines, it is also possible that ERK-mediated induction of MAPK phosphatases[8] results in underestimation of pathway stimulation in erythroid cells or that phospho-ERK staining in identifiable progenitors underestimates pathway activation in more primitive erythroid colony-forming cells.

These results also provide the first evidence that clinically achievable CI-1040 concentrations[5] inhibit MDS progenitors. While it is possible that cytokines added to support colony formation activated the MAPK pathway and enhanced CI-1040 sensitivity *in vitro*, our observation of pathway activation *in situ* (Figure 1) argues that exogenous cytokines are not activating a normally quiescent pathway. Several observations also suggest that CI-1040 might be inhibiting clonal precursors of MDS *in vitro*. First, effects of CI-1040 were greater in MDS marrow (Figure 2). Second, a marked reduction of clonally derived blast colonies was observed (Figures 2G,H). Because none of the MDS samples in the present study had informative cytogenetics, study of additional MDS samples is required to confirm the effects on clonal progenitors. Nonetheless, our preliminary results suggest that the MAPK pathway warrants further investigation as a potential drug target in MDS.

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Methodologic and biological variability of quantitative real-time polymerase chain reaction analysis of Bcr-Abl expression in Philadelphia chromosome-positive acute lymphoblastic leukemia

We examined to what extent technical and biological factors may affect the validity of Bcr-Abl polymerase chain reaction quantification in Philadelphia chromosome-positive acute lymphocytic leukemia and found that technical variance is the predominant limitation of the method and was not exceeded by biological variance. Interestingly, the number of $p210^{Bcr-Abl}$ (b2a2 or b3a2) transcripts per blast was more then 10-fold higher than the number of $p190^{Bcr-Abl}$ copies per blast.

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Bcr-Abl RNA transcripts[1] are suitable molecular markers for minimal residual disease (MRD) analysis in Philadelphia-positive acute lymphoblastic leukemia (Ph^+ALL) and may be used to guide therapy. However, MRD analysis by real time polymerase chain reaction (RT-PCR) may be affected by intra- and inter-assay variability as well as potentially by changes in Bcr-Abl copies per blast and differences between bone marrow (BM) and peripheral blood (PB).

PB and BM samples were obtained from 56 patients with relapsed or refractory Ph^+ ALL who were treated in phase II studies with imatinib (CSTI571 109 and CSTI571 114) as described previously.[2,3] Total RNA was extracted from mononuclear cells (Ambion) and cDNA synthesized from 1-5 μg RNA (RNA at 70°C for 10 min, 4°C until addition of core mix including 500 U SuperScript II RNase H- RT (Invitrogen), 50 U RNAguard (Amersham), 6.25 μM pDN_6 (Amersham), 1 mM dNTP and 10 mM DTT in 50 μL; then thermocycled at 25°C for 10 min, 42°C for 45 min and 99°C for 3 min) before undergoing column purification (Qiagen). Ten-fold plasmid standard dilutions with 10 to 10^6 copy numbers of Bcr-Abl and GAPDH (housekeeping gene) were analyzed simultaneously with patients' samples in duplicate reactions employing an ABI PRISM 7700 (Applied Biosystems) and Taqman PCR under standard conditions already described.[3] Bcr-Abl levels were calculated as the logarithm of the ratio between Bcr-Abl and GAPDH copies and, if appropriate, normalized to 100% leukemic blasts by cytology and flow cytometry at successive relapses. Statistical analysis included t-tests, ANOVA tests and F tests. Ranges including 90% of Gaussian distribution of intra- and inter-assay variability were calculated by the formula $\times$ +/- $t_{p,n-1}$. s ($\times$: mean; $t_{p,n-1}$: specific factor for n data points from table for t-values; s: standard deviation).[4]

The method permitted detection of 10^{-5} SD1/Nalm6 cells, 10^{-6} K562/Nalm6 and 10^{-4} BV173/Nalm6 cells. Leukemic blasts from patients (n=3, respectively) expressing e1a2, b2a2 or b3a2 were titrated into normal PB and BM cells. The average sensitivity of detection was 4×10^{-5}, 8×10^{-6} and 2×10^{-6} in BM samples and 6×10^{-6}, 7×10^{-7} and 4×10^{-6} in PB samples.

Intra-assay variability of SD1 cells (diluted 1:500) was ±0.20 log for RNA extraction plus cDNA synthesis plus PCR (n=5), ±0.17 log for separate cDNA synthesis plus PCR and ± 0.11 log for PCR only (differences not significant).

The inter-assay variability of patients' samples ranged from ±0.3 log at high MRD (log Bcr-Abl/GAPDH: -1.5) to ± 0.9 log at low levels (-5) and ± 1.4 log at a MRD level at the detection limit (-7) (Figure 1).

The correlation coefficient of paired PB and BM levels of e1a2, b2a2, or b3a2 was high (0.9, 0.85 and 0.93, respectively). Levels of Bcr-Abl were higher in BM than in PB: by 0.8 log for e1a2 ($p<0.001$), 0.3 log for b2a2 ($p=0.20$) and 0.7 log for b3a2 ($p=0.004$). Since Bcr-Abl levels normalized to 100%