Cancer Cell
Article

Single-Cell Profiling Identifies Aberrant STAT5 Activation in Myeloid Malignancies with Specific Clinical and Biologic Correlates

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DOI 10.1016/j.ccr.2008.08.014

SUMMARY

Progress in understanding the molecular pathogenesis of human myeloproliferative disorders (MPDs) has led to guidelines incorporating genetic assays with histopathology during diagnosis. Advances in flow cytometry have made it possible to simultaneously measure cell type and signaling abnormalities arising as a consequence of genetic pathologies. Using flow cytometry, we observed a specific evoked STAT5 signaling signature in a subset of samples from patients suspected of having juvenile myelomonocytic leukemia (JMML), an aggressive MPD with a challenging clinical presentation during active disease. This signature was a specific feature involving JAK-STAT signaling, suggesting a critical role of this pathway in the biological mechanism of this disorder and indicating potential targets for future therapies.

INTRODUCTION

Myeloproliferative disorders (MPDs) are clonal malignancies characterized by overproduction of immature and mature myeloid cells showing organ infiltration. In particular, juvenile myelomonocytic leukemia (JMML) and chronic myelomonocytic leukemia (CMML) are characterized by malignant transformation in the stem cell compartment with clonal proliferation of progeny that variably retain the capacity to differentiate (Arico et al., 1997; Onida et al., 2002). Children suspected of having JMML often present with failure to thrive, fever, infection, splenomegaly, and a high white blood cell count with monocytosis. Current diagnostic criteria are imprecise and consist of major and minor requirements that are in large part based on excluding other conditions (Niemeyer et al., 1997). The major requirements include an absolute monocytosis > 1,000/μl, fewer than 20% bone marrow blasts, and absence of the t(9;22) or *BCR-ABL* fusion gene. Patients must also meet two of the minor criteria, including an elevated fetal hemoglobin level for age, circulating myeloid precursors, a total white blood cell count > 10,000/μl, and in vitro hypersensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF).

Extensive molecular data implicate genetic lesions that deregulate Ras signaling as key initiating events in JMML, with studies showing that 60% of patients harbor an oncogenic mutation in *PTPN11*, *NRAS*, or *KRAS* while another 15% have clinical

SIGNIFICANCE

Recent advances have enabled simultaneous measurement of cell type and cell signals in primary populations using flow cytometry. This technique allows us to answer the question, "Can we track oncogenic cell populations from diagnosis through disease evolution via signaling?" Doing so in an era of using specific inhibitors against components of key signal transduction pathways will be necessary to assess treatment effects in human patients to further adapt therapies as cancer cells alter their signaling in response to these treatments. This work uses such an approach to follow patients over time and shows that disease status in juvenile myelomonocytic leukemia (JMML)—at diagnosis, remission, relapse, and transformation—is indicated by a subset of cells with an abnormal signaling profile.

neurofibromatosis type 1 and/or demonstrate loss of the wild-type *NF1* allele in their diseased bone marrow (Emanuel, 2004; Flotho et al., 2007). Patients with the myeloproliferative subtype of CMML exhibit *NRAS*, *KRAS*, and *JAK2* mutations (Levine et al., 2005; Onida et al., 2002). A cellular characteristic of both JMML and CMML is the formation of abnormal numbers of granulocyte-macrophage colony-forming units (CFU-GM) in methylcellulose cultures containing subsaturating concentrations of GM-CSF (Cambier et al., 1997; Emanuel et al., 1991), which has led to suggestions that alterations downstream of the activated GM-CSF receptor collaborate to drive inappropriate cell growth and survival. One important primary signaling event following binding of GM-CSF to the GM-CSF receptor is activation of the JAK-STAT pathway (Paukku and Silvennoinen, 2004). JAK2 transphosphorylates the β common chain of the GM-CSF receptor, which creates docking sites for adaptors and signal relay molecules, resulting in activation of Ras and downstream Ras effectors including ERK and S6 ribosomal protein (see Figure S1 available online) (Irish et al., 2004; Kunz and Ibrahim, 2003; McCubrey et al., 2000; Rane and Reddy, 2002; Shuai and Liu, 2003).

CMML is an adult MPD that is clinically similar to JMML and shares certain genetic features such the frequent presence of *RAS* mutations (Onida et al., 2002). *JAK2* mutations are rare in JMML and only slightly more common in CMML patients (Levine et al., 2005; Steensma et al., 2005; Zecca et al., 2007), whereas *PTPN11* mutations are almost nonexistent in CMML (Loh et al., 2005). Both JMML and CMML can progress to M4 or M5 acute myeloid leukemia (AML), which comprise the myelomonocytic (M4) and monocytic (M5) subtypes (Arico et al., 1997). Furthermore, somatic *NRAS*, *KRAS*, and *PTPN11* mutations occur frequently in the M4 and M5 subtypes of AML (Bacher et al., 2006; Loh et al., 2004).

Currently, it takes up to 3–4 weeks to confirm a suspected diagnosis of JMML with a CFU-GM assay. As early allogeneic hematopoietic stem cell transplant (HSCT) is the only potentially curative therapy for JMML (Locatelli et al., 2005), it is important to quickly and accurately diagnose these patients in order to deliver appropriate therapy in a timely fashion. In addition, monitoring disease burden during treatment is challenging in patients with JMML due to imprecise clinical definitions of response. Current allele-specific PCR methodologies to detect minimal residual disease are only applicable to approximately 60% of patients (Archambeault et al., 2008). Importantly, because JMML and CMML exhibit considerable cellular heterogeneity, it has been difficult to elucidate the biologic features of cells that contribute to the cancer phenotype in vivo and of precursor populations that might carry genetic lesions predisposing cells to an oncogenic fate.

Assays for identifying therapeutic agents and assessing efficacy in these patients based on the biochemical consequences of lesions in the GM-CSF and Ras signaling networks are few. Recent advances in flow cytometry, however, have made it possible to simultaneously measure cell type and aberrant cell signals (Irish et al., 2006) arising as a consequence of these lesions. We used this approach to profile signaling at the single-cell level (Irish et al., 2004; Van Meter et al., 2007), including molecules downstream of the GM-CSF receptor and molecules closely associated with Ras signaling, for the presence of primary JMML cells with altered signaling behavior that correlate with disease physiology. Our cohort of 52 samples included patients diagnosed with JMML, healthy individuals, infants with other MPDs, and children initially suspected of having JMML who were subsequently diagnosed with other disorders.

RESULTS

A Flow Cytometry-Based Signaling Assay Can Be Used to Measure GM-CSF Hypersensitivity

We used phosphospecific flow cytometry (Irish et al., 2004, 2006) after exposure to increasing concentrations of GM-CSF to interrogate evoked signaling responses in JMML cells. In a first test of GM-CSF-induced phosphorylation of STAT5, we observed the dose-dependent appearance of a population of cells in a JMML bone marrow sample compared to normal healthy bone marrow (Figure 1A). This leukemia was also assessed via the traditional methylcellulose assay and exhibited hypersensitive colony formation (defined as clusters of >50 cells) at increasing concentrations of GM-CSF (Figures 1C and 1D), as described previously (Emanuel et al., 1991).

We then investigated 11 additional JMML samples at diagnosis and compared these leukemias to normal samples (n = 8), other childhood MPDs (these cases included 8 patients with Noonan syndrome/MPD [NS/MPD] or Down's syndrome with transient myeloproliferative disorders [DS/TMD]), and 4 children with an initial clinical suspicion of JMML who were subsequently found to have another diagnosis. We observed an induced phosphorylated STAT5 (p-STAT5) population in the majority of JMML samples that were exposed to low levels of GM-CSF, but not in the other samples interrogated. The data were quantified as a relative percentage of p-STAT5-responsive cells as outlined in the Experimental Procedures (Figure 1B).

p-STAT5 Response to Low Doses of GM-CSF Indicates JMML Status

The combination of CD38 and p-STAT5 best stratified the GM-CSF-hyperresponsive population, which was measurable in both peripheral blood and bone marrow samples. Representative samples from the patient cohort are shown in Figure 2A. The signature was present in both fresh and previously frozen primary samples (details for each sample are provided in Table S1).

p-STAT5 Signaling Cells in JMML Samples Are of Myeloid Origin and Require JAK2 Activity

Immunophenotyping revealed that the p-STAT5-responsive cells were of myeloid origin ($CD33^{+}CD14^{+}$), $CD34^{-}$, and $CD38^{lo}$ (Figure S2). The involvement of JAK-specific activation of the p-STAT5 response in these cells was first confirmed by exposing primary samples to a chemical JAK2 inhibitor for 30 min before GM-CSF stimulation at 10 ng/ml for 15 min (Figure S3). Similarly, exposing JMML cells to a 5 μM concentration of the oral JAK2 inhibitor XL019 (Exelixis) inhibited STAT5 and ERK phosphorylation in response to a saturating concentration of GM-CSF, whereas the MEK inhibitor CI-1040 (Pfizer) failed to alter the p-STAT5 response despite inhibiting p-ERK (Figure 2B).