Experimental Hematology

Experimental Hematology 2014;42:360–368

Cytokine profiles in polycythemia vera and essential thrombocythemia patients: Clinical implications

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(Received 10 April 2013; revised 10 January 2014; accepted 17 January 2014)

Studies have shown that the clinical impact of Janus kinase 2 (JAK2) inhibitors in primary myelofibrosis patients is due to the regulation of cytokine levels, suggesting that cytokine profiles might play a critical role in myeloproliferative neoplasms (MPNs) physiopathology. In this study, we compared the plasma cytokine profiles of polycythemia vera (PV) patients and essential thrombocythemia (ET) patients as a function of their JAK2 V617F status and the presence of thrombohemorrhagic complications. Using a multiplex cytokine assay, cytokine measurements were taken of the plasma of 17 PV patients and 21 ET patients. Twenty-two of these patients (10 PV and 12 ET) experienced at least one thrombohemorrhagic manifestation before diagnosis. We showed that cytokine levels were significantly increased in PV and ET patients compared with normal values and that several positive correlations existed between the cytokine concentrations and the biological parameters in each MPN. The comparison between the cytokine profiles of ET and PV patients showed a statistically significant increase of interleukin (IL)-4, IL-8, granulocyte macrophage–colony stimulating factor, interferon -γ, monocyte chemotactic protein -1, platelet derived growth factor-BB, and vascular endothelial growth factor in the ET group. Only tumor necrosis factor-α and platelet derived growth factor-BB were specifically impacted by the JAK2 V617F status of the PV and ET patients, respectively, suggesting that there are both JAK2 V617F–driven and JAK2 V617F–independent inflammatory responses in MPNs. We also showed that the subgroup of PV patients with vascular complications displayed significantly different concentrations of IL-12(p70) and granulocyte macrophage–colony stimulating factor compared with patients without vascular complications. Altogether, these data suggest that cytokine measurement might be useful for the clinical and therapeutic stratification of PV and ET patients. © **2014 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Myeloproliferative neoplasms (MPNs) are hemopathies in which an acquired hematopoietic stem cell alteration leads to a deregulated production of peripheral blood cells by the bone marrow [1], which results in abnormal levels of red blood cells, leukocytes, or thrombocytes. Polycythemia vera (PV) and essential thrombocythemia (ET) are the most common Philadelphia chromosome negative MPNs . PV and ET are characterized by an increased red cell mass and thrombocyte count, respectively, associated or not with an increase of the leukocyte count [2]. These disorders may evolve into myelofibrosis or, less commonly, into acute leukemia [3,4], but the primary causes of morbidity and mortality in MPN patients are thrombotic complications and, to a lesser degree, bleeding [3–6]. In fact, PV and ET are associated with a significantly increased risk of arterial or venous thrombosis and bleeding complications [7].

The discovery of a gain-of-function mutation of Janus kinase 2 (JAK2 V617F) and its role in MPNs [8–10] has focused considerable attention on JAK2 inhibitors as potential therapeutic targets. Clinical studies of primary myelofibrosis (PMF) have highlighted an unexpected impact of

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0301-472X/$ - see front matter.
http://dx.doi.org/10.1016/j.exphem.2014.01.006

these inhibitors. JAK2 inhibitors primarily act on constitutional symptoms (asthenia, cachexia, satiety, night sweats), thereby improving a patient's quality of life. The inhibitors also mitigate the symptoms of splenomegaly, including reductions in spleen size and the attenuation of symptoms such as abdominal discomfort or early satiety [11–14], without evidence of the regulation of the JAK2 V617F allele burden. Studies have shown that this clinical impact is partially due to the down regulation of cytokine levels. In fact, PMF patients harbor increased levels of the following cytokines, which are all significantly reduced by treatment with JAK1/JAK2 inhibitors: the antagonist receptor of interleukin-1 (IL-1RA), macrophage inflammatory protein (MIP)-1β, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), interleukin (IL)-6 and IL-8 [12].

These data have highlighted new issues regarding the potential physiopathologic role of plasma cytokines in MPNs. The hypersensitivity of the erythroid progenitors to erythropoietin (Epo) is a well-known hallmark of PV, and erythroid colonies can be generated in vitro in the absence of exogenous cytokines. The variations of plasma Epo levels can enable the differentiation between primary and secondary erythrocytosis; however, these variations are only the consequence of autonomous erythroid growth and are not related to the clinical phenotype. Boissinot et al. provided evidence that inflammation-linked cytokines were required for the growth of JAK2 V617F–mutated erythroid progenitors [15]. On the other hand, anti-inflammatory hepatocyte growth factor (HGF) and IL-11 were also upregulated in PV, and both cytokines contributed to the proliferation of PV erythroblasts [15]. Moreover, Tefferi et al. showed that IL-8, IL-2R, IL-12, IL-15 and interferon-inducible protein (IP)-10 were independent predictive factors of inferior quality of life in PMF patients and that some phenotype cytokine associations could be highlighted [16]. It is also noteworthy that the capacity of some cytokines to modulate hemostasis plays a role in the manifestation of thrombotic events, and one could thereby hypothesize that they might also contribute to the vascular complications of MPNs. Altogether, these data encourage further clinical evaluation of plasma cytokines in PV and ET.

For this reason, we conducted a comparative study of the plasma cytokine profiles of MPN patients using a multiplex immunoassay as a function of PV, ET, JAK2 V617F status, and the presence (or not) of a thrombohemorrhagic complication before the diagnosis.

Methods

Patients and sample preparation

Thirty-eight patients diagnosed with a myeloproliferative neoplasm between 2004 and 2007 entered the study at the University Hospital of Grenoble (France). Seventeen patients had polycythemia vera, and 21 patients had essential thrombocythemia. Approvals and informed consent were obtained according to the Declaration of Helsinki. Diagnosis was established based on the pre-2008 group criteria for polycythemia vera and was verified by the 2008 World Health Organization criteria. All PV patients without the JAK2 V617F mutation were positive for endogenous erythroid colonies, displayed low Epo serum values, and exhibited no primary cause for polycythemia. To eliminate a diagnosis of prefibrotic PMF, the following biological parameters were verified in the ET patients: absence of fibrous tissue from osteomedullar biopsy and absence of erythroblast myelemia, cytopenia, or cell morphology disorders such as dacryocytosis. To study the impact of previous vascular events on the cytokine profile, we selected PV and ET patients that fit into one of the following two subgroups: patients with previous vascular events ($n = 22$) or patients without vascular complications ($n = 16$). Similarly, we selected a significant number of JAK2 V617 negative patients ($n = 14$) for comparison with JAK2 V617F positive patients ($n = 24$). These criteria explain the relatively low percentage of JAK2 V617F positive PV patients (76%) in our cohort compared with the well-known general frequency of the mutation in PV (95%). Peripheral blood (plasma from a citrate tube) was collected upon diagnosis of MPN, so none of the patients were on therapy when the samples were stored. Samples were spun at 2,000 rpm for 10 min (Multifuge Kandro 3S/3S-R, Heraeus, Germany), and plasma was collected and stored at −80°C until testing.

Evaluation of plasmatic cytokine levels

Cytokine levels were measured using the xMAP immunoassay technology with formatted magnetic beads. Thirteen cytokines (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12(p70), granulocyte macrophage–colony stimulating factor [GM-CSF], interferon [IFN]-γ, monocyte chemotactic protein [MCP]-1, platelet derived growth factor [PDGF]-BB, TNF-α, VEGF, and HGF) were evaluated by Bio-Plex Pro Assays (Bio-Rad, Marnes La Coquette, France). All experiments were performed according to the manufacturer's protocol. All experiments were run in duplicate.

Fifty micrograms of each type of 13 coupled beads, which corresponded to each cytokine being studied, were added to each well of a 96-well plate. Beads were washed twice, 50 μL of the eight standard set (mix of cytokines provide by Bio-Rad and for which concentration are known) and patient's plasma samples were diluted 1:4, added to the wells, and incubated for 30 min with shaking at room temperature. After washing, 25 μL of diluted detection antibodies were added and incubated for another 30 min. Finally, 50 μL of diluted streptavidin-phycoerythrin was added, and the wells were washed three times before adding 125 μL of assay buffer to each well. The plate was read with the Bio-Plex200 System (Bio-Rad), and data acquisition and analysis were accomplished with the Bio-Plex Manager 5.0 (Bio-Rad) software at a low photomultiplier tubes. The fluorescence signal of a minimum of 50 beads per cytokine was evaluated and recorded. The concentration (pg/mL) of each cytokine was calculated from its standard curve.

Statistical analysis

All quantitative variables were expressed as medians. The comparison of the data was carried out using the Mann Whitney t test. Correlations between the cytokine levels and the main biological parameters were investigated by the Spearman test. Statistical analyses were performed using StatView 5.0 (SAS Institute,