Perspectives on thrombosis in essential thrombocythemia and polycythemia vera: is leukocytosis a causative factor?

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Leukocyte (WBC) count has been recently identified as an independent predictor of major thrombosis in both essential thrombocythemia (ET) and polycythemia vera (PV). However, whether leukocytosis should be simply considered a marker for vascular disease or whether elevated WBC levels actually contribute directly to causing such disorders is presently matter of many studies. By adopting epidemiologic criteria for causation, we have examined the characteristics to support this association such as (1) strength, (2) consistency, (3) specificity, (4) temporality, (5) biologic gradient, (6) plausibility, (7) experimental evidence, and (8) analogy. Our conclusion supports the notion that baseline leukocytosis in ET and PV patients adds prognostic significance to existing risk factors and that may be considered causative of vascular events. These developments could induce clinicians to incorporate WBC count into standard clinical practice. However, we need prospective clinical studies with stratification of patients according to their baseline leukocyte counts. Until such evidence is available, the decision on how to manage these patients should continue to follow conventional criteria. (Blood. 2009;114:759-763)

Introduction

The clinical course of essential thrombocythemia (ET) and polycythemia vera (PV) is characterized by an increased incidence of vascular complications and a tendency to progress into myelofibrosis or acute myeloid leukemia. In the pathogenesis of thrombosis, more than one cause is involved including patient- and disease-related factors. It is widely accepted that age and previous thrombotic events are risk factors for new major vascular complications both in ET and PV. On this basis, patients are now stratified into low risk and high risk and the use of cytoreductive drugs is recommended in the high-risk category. On the contrary, there is still much controversy about the role played by conventional vascular risk factors such as diabetes, hypertension, and smoking since many multivariable analyses did not consistently demonstrate their independent pathogenetic role.

More recently, disease-related risk factors have been considered, including the presence of JAK2V617F mutation and baseline leukocyte (WBC) count. The latter has been found to be an independent predictor of total major thrombosis, particularly acute coronary syndromes, both in ET and PV. However, whether leukocytosis is simply a marker for vascular disease or whether elevated WBC levels actually contribute directly to causing such disorders is presently unknown. This question has clinical importance for 2 reasons: the first is that new patient stratification, based on baseline leukocytosis, could be proposed in future clinical trials, and the second is that WBCs could be a target of therapy.

We attempted here to recognize the role of causality for major vascular events played by leukocytes in ET and, to this end, we reviewed the pertinent literature and adopted the epidemiologic criteria proposed by Hill. The following characteristics to support this association were examined: (1) strength, (2) consistency, (3) specificity, (4) temporality, (5) biologic gradient, (6) plausibility, (7) experimental evidence, and (8) analogy.

Strength

The incidence of major thrombosis and risk factors were examined in a large retrospective cohort of 1063 ET patients in whom it was ascertained the role of patient-related (age and previous vascular events) and disease-related risk determinants, including platelet and leukocyte counts, and JAK2V617F mutation and allele burden.

A total of 193 patients (18%) had a prior history of thrombosis, which was made up of an arterial event in 133 (69%) and venous thrombosis in 60 (31%). Patients were classified as being at low or high risk for thrombosis according to standard risk factors (age ≥60 years and/or a previous major thrombotic event). Low-risk patients (n = 517, 49%) were followed with no cytoreductive therapy, whereas high-risk patients (n = 546, 51%) were given hydroxyurea (HU) in the great majority (90%) of cases and busulfan in a small group of elderly patients (5%). The target of therapy was to keep platelet number less than 600 × 10^9/L. Low-dose aspirin (100 mg daily) was prescribed in 703 patients (66%) according to the indication of the physician in charge.

During up to 38 years of follow-up (median, 4.8 years), 118 major thrombosis (2.3% patients/year) were objectively diagnosed and included 48 ischemic cerebral strokes or transient ischemic attacks (TIA), 25 acute myocardial infarction (MI), 11 peripheral arterial disease (PAT), and 34 venous thromboembolism (TE). Multivariable analysis confirmed that age and previous thrombosis were independent factors for occlusive events (HR = 1.7, 95% CI = 1.1-2.6, P = .01).

In regard to disease-related risk factors, we examined the predictive role of baseline leukocyte levels (Table 1). Compared with patients with WBC count less than 8 × 10^9/L (n = 391; 37%), those with WBC count higher than 11 × 10^9/L (n = 220; 21%) had
Table 1. Multivariable analysis on the relative risk of major thrombosis among 1063 ET patients

<table>
<thead>
<tr>
<th>Major thrombosis</th>
<th>8.0-11.0 x 10^9/L</th>
<th>More than 11.0 x 10^9/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>(1) Unadjusted</td>
<td>1.3 (0.8-1.9)</td>
<td>.3</td>
</tr>
<tr>
<td>(2) Variables adjusted</td>
<td>1.4 (0.9-2.1)</td>
<td>.2</td>
</tr>
<tr>
<td>(3) + JAK2V617F adjusted</td>
<td>1.5 (0.9-2.5)</td>
<td>.1</td>
</tr>
</tbody>
</table>

Model 1: platelet count (3 categories) or white blood cell count (3 categories).
Model 2: model 1 plus center (3 categories), sex (2 categories), standard risk factors: age 60 years or older and/or previous thrombotic event (yes/no), hemoglobin at diagnosis (2 categories, median value used as cutoff), white blood cell count or platelet count at diagnosis (2 categories, median value used as cutoff), antiplatelet use (yes/no), chemotherapy use (yes/no).
Model 3: model 2 plus JAK2V617F status (yes/no).
*Reference categories: white blood cell count lower than 8 x 10^9/L. Cutoffs arbitrarily defined.

Table 2. Interaction of leukocyte and platelet counts at diagnosis (multivariable model)

<table>
<thead>
<tr>
<th>Interaction, value x 10^9/L</th>
<th>n (%)*</th>
<th>Rate of vascular event</th>
<th>Low risk† (%)</th>
<th>JAK2V617F‡ (%)</th>
<th>RR (P) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) WBC &lt; 11 + PLT &gt; 1000</td>
<td>170 (16)</td>
<td>1.59</td>
<td>95 (56)</td>
<td>35 (26)</td>
<td>1</td>
</tr>
<tr>
<td>(2) WBC &lt; 11 + PLT &lt; 1000</td>
<td>644 (62)</td>
<td>2.26</td>
<td>317 (49)</td>
<td>304 (56)</td>
<td>1.92 (.034) (1.07-2.87)</td>
</tr>
<tr>
<td>(3) WBC &gt; 11 + PLT &gt; 1000</td>
<td>99 (9)</td>
<td>2.88</td>
<td>41 (41)</td>
<td>40 (61)</td>
<td>2.38 (.026) (1.11-3.51)</td>
</tr>
<tr>
<td>(4) WBC &gt; 11 + PLT &lt; 1000</td>
<td>130 (12)</td>
<td>2.95</td>
<td>52 (40)</td>
<td>75 (77)</td>
<td>2.43 (.017) (1.25-2.94)</td>
</tr>
</tbody>
</table>

*aTwenty patients were excluded because of WBC or platelet count data missing at diagnosis.
†Percentage of patients per year.
‡Age younger than 60 years and no previous thrombosis; untreated (90%).
§Percentage calculated on 860 patients with JAK2V617F status evaluated.

Table 3. Time-dependent multivariable analysis on the relative risk of major thrombosis in ECLAP study (N = 1638)

<table>
<thead>
<tr>
<th>White blood cell count, x 10^9/L</th>
<th>Hazard ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 10 (n = 990)</td>
<td>1 (reference)</td>
<td></td>
</tr>
<tr>
<td>10.1-15 (n = 365)</td>
<td>1.06 (0.7-1.6)</td>
<td>.8</td>
</tr>
<tr>
<td>&gt;15 (n = 241)</td>
<td>1.71 (1.1-2.6)</td>
<td>.02</td>
</tr>
</tbody>
</table>

*Model adjusted for: age, sex, time from PV diagnosis to recruitment, thrombotic or hemorrhagic events prior to recruitment, smoking, history of diabetes, hypertension, claudicatio intermittens, erythromelalgia, splenomegaly, circulating immature cells, leukocyte count, total blood cholesterol, phlebotomy use, interferon use, hydroxyurea use, antiplatelet use, anticoagulant use, 32P use, busulfan use, chlorambucil use, and pipobroman use.

a significantly higher risk of major thrombosis both in unadjusted and in the progressively adjusted predictive models.

To see whether the prognostic risk of thrombosis was driven not only by individual WBC count but also by interaction with platelet count, we analyzed 4 groups of patients according to their baseline leukocyte count (lower or greater than 11 x 10^9/L) and platelet count (lower or greater than 1000 x 10^9/L; Table 2). Considering patients presenting with WBC count lower than 11 x 10^9/L and extreme thrombocytosis as the reference group, the incidence and the relative risk of thrombosis progressively increased, as well as the proportion of JAK2V617F mutated patients (up to 77%). Indeed, thrombocytosis was significantly associated with a better prognosis only in patients with low WBC count (groups 1 and 2), whereas in patients with leukocytosis (groups 3 and 4) the effect of platelet count was not more significant (P = .126). No difference across groups was demonstrated in terms of conventional risk factor distribution and percentage of JAK2V617F allele burden mutation.

Leukocytosis was able to add prognostic significance to existing risk factors. This was demonstrated in younger and asymptomatic cases by C-statistic index. In this low-risk category, which accounted for 52% of our patients, C statistic was slightly higher (C = 0.65) than that calculated in conventional high-risk group (C = 0.60), indicating that leukocytosis per se can discriminate 2 subgroups of patients within the low-risk group who are at different risk of future vascular complications. Thus, on the basis of baseline leukocytosis, we suggest to re-evaluate risk stratification in patients with ET conventionally considered to be at low risk for cardiovascular events. From these data, the independent association between baseline leukocytosis and vascular events appears to be strong and the hypothesis that this association is entirely due to other unmeasured confounders or other source of bias may be reasonably ruled out.

Leukocytosis was found to be an independent risk factor for thrombosis in PV as well. Time-dependent multivariate analysis, adjusted for potential confounders including cytoreductive and antithrombotic treatment, demonstrated that patients participating in the ECLAP study with a leukocyte count greater than 15 x 10^9/L had a significant increase in the risk of major thrombosis compared with patients with leukocyte lower than 10 x 10^9/L (HR = 1.71, 95% CI = 1.1-2.6; Table 3).

Consistency

The association between baseline leukocytosis and thrombosis appears to be consistent. Wolanskyj et al reported that leukocyte counts higher than 15 x 10^9/L were independent predictors of major thrombotic events (RR = 1.74, 95% CI = 1.15-2.66) in a retrospective study including a large number of patients (N = 322) with ET selected on the basis of at least a decade of potential follow-up after diagnosis. The same investigators repeated this analysis in their whole, unslected population of 605 ET patients, who differed from the previous selected group for a shorter follow-up median (84 months), and did not confirm the predictive role of leukocytosis for incident venous thrombotic events (P = .73). In contrast, the association of leukocytosis and arterial thrombosis had a borderline significance (P = .09). The different result of these studies might suggest that time duration of exposure to leukocytosis may have a role for the development of vascular complications. This concept is in keeping with the putative mechanisms of atherogenesis and thrombogenesis associated with leukocytosis, described in “Plausibility.” In addition, it should be noted that the relationship between leukocyte count at diagnosis and subsequent thrombosis may be confounded by treatments, which could be variable in the different series.

This issue was tackled by other investigators. Caramazza et al found that white blood cell (WBC) counts higher than 9.5 x 10^9/L at diagnosis were independently associated with thrombosis during...
follow-up (RR = 1.8, P = .03). At the time-dependent analysis, therapy with hydroxyurea (HU), lowering by 35% the baseline WBC level, reduced such strength of association giving an RR of 1.3 (P value nonsignificant). Similar results supporting the relationship between baseline leukocytosis and thrombosis were reported by others.16

### Specificity

The criterion of specificity requires that a cause leads to a single and not multiple effects. The role of WBC count in ET was mostly observed on myocardial infarction occurrence, as reported in Table 4, thus suggesting some specificity for this event.4 Interestingly, the same observation was made in patients with PV enrolled in the ECLAP study (European Collaboration in Low-Dose Aspirin in Polycythemia Vera), in which patients with WBC count higher than 15 × 10⁹/L had a significant increase of myocardial infarction risk (HR = 2.84; 95% CI = 1.25-6.46, P = .01).9

### Temporality

Temporality refers to the necessity for a cause to precede an effect in time. This fourth characteristic might be particularly relevant with diseases of slow development such as atherosclerosis in ET and PV. We have explored this characteristic in a study of 439 patients with ET.7 Two statistical multivariable analyses were performed considering variables at diagnosis and before the vascular events occurred during follow-up. The multivariable “baseline” analysis showed a significant and independent association between high levels of white blood cells with the occurrence of thrombosis in follow-up (HR = 2.3, 95% CI: 2.4-3.9). On multivariable time-dependent analysis, we took into account the last value of blood cell counts measured before vascular events. Treatment with hydroxyurea decreased the leukocyte count to about 30%, from the baseline median level of 8.7 × 10⁹/L to 6.1 × 10⁹/L, and for this reason untreated low-risk patients with high levels of leukocytes showed a vascular risk similar to that observed in high-risk patients with normal leukocyte count (HR = 3.1, 95% CI = 1.4-7.1 and HR = 2.5, 95% CI = 1.0-6.0, respectively).

The criterion of temporality is satisfied also in PV since the significant association between leukocytosis and thrombosis found in the ECLAP study9 was observed by a multivariable time-dependent analysis.

### Biologic gradient

Biologic gradient refers to the presence of a unidirectional dose-response curve between cause and effect. In our series of ET

### Plausibility

Arterial and venous thrombosis are multifactorial diseases and are associated with multiple interacting genetic and environmental risk factors. For arterial disease, some of these risk factors can be “atherogenic” promoting progression of occlusive atherosclerosis (eg, smoking, hypertension, cholesterol, diabetes), whereas others may be “thrombogenic” (eg, obesity, diabetes, immobility, estrogens, infections, smoking) promoting rupture of atheromatous plaques and superadded thrombosis. There is now increasing evidence that arterial disease may directly promote venous thromboembolic events17 and that activation of inflammation and hemostatic system plays a role in progression of atherosclerosis.18 In this context, laboratory and epidemiologic investigations have attributed a major pathogenetic role to leukocytes both in terms of promoting atherogenesis and thrombogenesis.19 Thus, it is not surprising that a long-term exposition of activated leukocytes, as shown in these myeloproliferative neoplasms (MPNs), can damage vascular endothelium and promote thrombosis. Mechanisms of this action involve multiple interactions with platelets and vessel walls as shown by many laboratory studies.20,21 Polymorphonucleated neutrophils (PMNs) can release reactive oxygen species and intracellular proteases, which can act on endothelial cells and platelets and may modify the hemostatic balance toward a prothrombotic state. Indeed leukocyte elastase and cathepsin G can induce detachment or lysis of endothelial cells and can also modify endothelial cell functions involved in thromboregulation.22 Furthermore, the potential thrombogenic effects of PMN-derived proteases include the direct potent platelet activation elicited by cathepsin G, and the augmentation of adhesion of neutrophils may affect neutrophil/platelet interaction leading to a significant increase in circulating neutrophil/platelet aggregates.23 Finally, PMN-derived elastase can directly proteolyze and inactivate natural inhibitors of blood coagulation, including protein C, protein S, tissue factor pathway inhibitor, antithrombin, and heparin cofactor II, thus impairing potent physiological antithrombotic mechanisms.24

### Experiment

Experimental evidence would result from intervention studies demonstrating that removal of leukocytosis exposure leads to a significant reduction of vascular events. This requires specifically designed prospective clinical trials. Nevertheless, it should be

### Table 4. Sites of thrombosis according to leukocytosis at diagnosis in 657 patients with ET9

<table>
<thead>
<tr>
<th>Leukocytosis Level</th>
<th>Major Thrombosis</th>
<th>Arterial Thrombosis</th>
<th>Myocardial Infarction (IMA)</th>
<th>Stroke/TIA</th>
<th>Venous Thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1-10 × 10⁹/L white blood cells*</td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
<td>P</td>
<td>HR (95% CI)</td>
</tr>
<tr>
<td>Major thrombosis</td>
<td>2.21 (1.05-4.65)</td>
<td>.036</td>
<td>3.27 (1.54-6.95)</td>
<td>.002</td>
<td>1.61 (1.05-2.46)</td>
</tr>
<tr>
<td>Arterial thrombosis</td>
<td>2.07 (0.63-6.92)</td>
<td>.121</td>
<td>3.12 (1.20-8.08)</td>
<td>.019</td>
<td>1.32 (0.61-2.86)</td>
</tr>
<tr>
<td>Myocardial infarction (IMA)</td>
<td>5.82 (2.64-12.32)</td>
<td>.118</td>
<td>8.08 (1.50-65.5)</td>
<td>.050</td>
<td>1.46 (0.75-2.85)</td>
</tr>
<tr>
<td>Stroke/TIA</td>
<td>0.89 (0.29-2.67)</td>
<td>.824</td>
<td>1.32 (0.42-4.11)</td>
<td>.631</td>
<td>2.51 (0.86-7.29)</td>
</tr>
<tr>
<td>Venous thrombosis</td>
<td>1.40 (0.49-4.04)</td>
<td>.534</td>
<td>2.51 (0.86-7.29)</td>
<td>.092</td>
<td>3.47 (1.15-10.48)</td>
</tr>
</tbody>
</table>

Multivariable models adjusted for information collected at diagnosis, including center, sex, standard risk factors, hemoglobin, hematocrit, and platelet count.

*Reference category: WBC count 7 × 10⁹/L or lower.
noted that myelosuppressive agents affecting also the granulocyte lineage are more effective in reducing thrombosis in ET than drugs inhibiting selectively the megakaryocyte maturation. In a randomized clinical trial comparing HU plus aspirin versus anagrelide plus aspirin in 809 patients with ET and a high risk of thrombosis, the median leukocyte count in the HU group was significantly lower and persistently lower than that in the anagrelide group (P < .001), whereas the control of platelet count was similar. When individual end points were assessed, coronary and cerebral arterial thrombosis were significantly less frequent in patients treated with HU (P = .004).

Interferon-alpha showed a broad myelosuppressive activity and a probable selective effect on mutated clones, as shown by complete JAK2V617F molecular remission in most PV patients. It has been underlined that the selective effect of IFN-alpha on mutated granulocytes may affect the incidence of thrombosis, as leukocyte activation and hemostatic changes are correlated with the presence of JAK2 mutation. Accordingly, the incidence of thrombosis in IFN-alpha–treated patients was consistently found to be lower than expected during follow-up.

These observations raise the question of how to consider leukocytes in the design of future clinical trials comparing different treatments for these disorders, including the new JAK2 inhibitors. This issue has been recently tackled in a consensus conference of European experts who convened to develop a definition of response to treatment in PV and ET. Achievement of leukocyte count lower than 10 × 10⁹/L was included among the criteria required for defining a complete clinicohematologic response in both diseases.38

Analogy

The association between leukocytosis and increased morbidity and mortality of ischemic vascular disease has been observed in the general population for more than half a century, and recent studies in 350,000 patients confirm the strength of the association and the dramatically higher mortality rates in patients with high versus low leukocyte counts. Another example showing a role of leukocytosis in vascular complication is the sickle cell disease (SCD) in which evidence that PMNs could contribute to poor prognosis and vaso-occlusive events has been provided. In SCD patients, good clinical response to hydroxyurea usually follows significant reduction in neutrophil count, even in the absence of other effects of the drug, such as rise in HbF level.

Conclusions

The bulk of information retrieved in the scientific literature regarding the association of leukocytosis and thrombosis fulfils the standards of epidemiologic evidence for causation established by the Hill criteria. However, there is an ongoing debate on how to interpret these criteria. According to Kundi, there are no criteria in the strict sense for the assessment of evidence concerning a factor’s propensity to cause a disease. This author pointed out that, starting from epidemiologic evidence, a factor may be attributed the potential of disease causation if there are no valid counterarguments to refute the hypothesis and that, even though every verdict of causation is provisional, action should not be postponed until better evidence is available. In regard to leukocytosis and thrombosis in ET and PV, the significance of the association appears to be supported specifically by the coherence of many results derived from independent prospective and retrospective cohorts and data sources, and there are no solid counterarguments to refute this notion so far. Thus, leukocytosis appears to be a potential causative factor of thrombosis and this concept raises the important question of how clinicians should use leukocyte count to classify patients with MPN according to the expected risk of vascular complications. In this regard, it should be kept in mind that causal inferences cannot attain the certainty of rigorous experimental studies. Therefore, the next step should be the organization of prospective clinical trials with baseline stratification of patients on the basis of leukocyte count. This would allow to test explicitly predefined hypotheses for subgroup analyses, aiming to explore the “causative” role of leukocyte number, such as the specific expression of a common more aggressive background condition or the definition of a subpopulation of poor responders. It seems paradoxical that leukocytes play a pivotal role in diseases characterized by proliferation mainly of platelets and red blood cells, but it is becoming clear, from molecular and clinical studies, that overall myeloproliferation rather than single cell lines should be the true target of future therapy in MPNs.

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Authorship

Contribution: T.B. designed and supervised the research project, wrote the paper, and raised funds; A.C. performed research, analyzed and interpreted data, and wrote the paper; A.R. performed research and raised funds; and G.F. designed and performed research, and wrote the paper.

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References

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