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The Regulation of Normal and Neoplastic Hematopoiesis is Dependent on Microenvironmental Cells.

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Abstract

Each day the adult human produces 4×10^{11} red blood cells, 1×10^{11} white blood cells and 1×10^{11} platelets, levels of production which can increase 10–20 fold in times of heightened demand. Hematopoiesis, or the formation of the ten different types of blood and marrow cells, is a complex process involving hematopoietic stem cells (HSCs), cytokine growth factors and cell surface adhesion molecules, and both specific and ubiquitous transcription factors. The marrow micro-environmental niche is defined as the site at which HSCs reside and are nurtured, receiving the signals that lead to their survival, replication and/or differentiation. Using microscopic, biochemical and molecular methods many different cells and the signals responsible for niche function have been identified. Early studies suggested two distinct anatomical sites for the niche, perivascular and periosteal, but the preponderance of evidence now favors the former. Within the "vascular niche" much evidence exists for important contributions by vascular endothelial cells (ECs), CXCL12-abundant reticular (CAR) cells and mesenchymal stromal cells, through their elaboration of chemokines, cytokines and cell surface adhesion molecules. In a series of studies we have found, and will present the evidence that megakaryocytes (MKs), the precursors of blood platelets, must be added to this list.

In addition to normal blood cell development, numerous studies have implicated the perivascular niche as contributing to the pathogenesis of a variety of hematological malignancies. Our laboratory focuses on the Ph^1-negative myeloproliferative neoplasms (MPNs), polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). These diseases are characterized by clonal expansion of HSCs and one or more mature blood cell types, hypermetabolism, a propensity to disorders of hemostasis (thrombosis>bleeding) and in some, evolution to acute leukemia. While a variety of therapies can control the abnormal expansion of the progeny of the malignant HSC, the only curative therapy is myeloablation with conditioning therapy or immunological means, followed by allogeneic stem cell transplantation (SCT), a procedure that is often inadequate due to relapse of the malignant clone. While the three disorders were postulated by Dameshek in the 1950s to be related to one another, proof came in 2005 when an acquired mutation in the signaling kinase Janus kinase 2 ($Jak2V_{617}F$) was identified in virtually all patients with PV, and ~50% of patients with ET and PMF. Since that time a number of other mutations have been identified that account for the "$Jak2V_{617}F$ negative" MPNs, including the

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thrombopoietin receptor, c-MPL, other mutations of Jak2, calreticulin and a variety of epigenetic modifier genes (e.g. TET2). Using a cell-specific Cre recombinase and SCT techniques we can introduce Jak2V_{617}F into murine megakaryocytes and platelets, hematopoietic stem cells, and endothelial cells, alone or in combination, in order to probe the role of the mutant kinase in various cells on several aspects of the MPNs. Using these tools we have found that the expression of Jak2V_{617}F in HSCs and ECs drives a MPN characterized by neutrophilia, thrombocytosis and splenomegaly, eventually evolving into myelosclerosis. Somewhat surprisingly, we found that Jak2V_{617}F-bearing ECs were required for many features of the MPN, such as enhancing the growth of Jak2V_{617}F-bearing HSCs over that of wild type HSCs, its characteristic radioresistance, and a hemostatic defect. Altogether, our studies suggest that the malignant vascular niche is a critical element in the pathogenesis of MPNs, and a more thorough understanding of the molecular basis for these findings could lead to improved treatment for patients with these disorders.

Introduction

The marrow micro-environment, or “niche” is defined as the site at which hematopoietic stem cells (HSCs) reside and are nurtured, receiving the signals that lead to their survival, replication and/or differentiation into all the mature cells of the blood. Using microscopic, biochemical and molecular methods many different cells and the signals responsible for niche function have been identified. Early studies suggested two distinct anatomical sites for the niche, perivascular and periosteal, but the preponderance of evidence now favors the former[1]. Within the “vascular niche” much evidence exists for important contributions by vascular endothelial cells (ECs), CXCL12-abundant reticular (CAR) cells and mesenchymal stromal cells, through their elaboration of chemokines, cytokines and cell surface adhesion molecules. In a series of studies we have found, and will present the evidence that megakaryocytes (MKs), the precursors of blood platelets, must be added to this list.

In addition to normal blood cell development, numerous studies have implicated the perivascular niche as contributing to the pathogenesis of a variety of hematological malignancies[2]. Our laboratory focuses on the Ph^1-negative myeloproliferative neoplasms (MPNs), which includes polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). These disorders are not rare, together affecting approximately 1 in 1000 individuals in the United States, are characterized by clonal expansion of HSCs and one or more mature blood cell types, hypermetabolism, a propensity to disorders of hemostasis (thrombosis>bleeding) and in some, especially if treated with genotoxic agents to control blood cell counts, evolution to acute leukemia. While a variety of therapies can control the abnormal expansion of the progeny of the malignant HSC, the only curative therapy is stem cell transplantation (SCT), a procedure that is rather toxic and often inadequate due to relapse of the malignant clone.

While the three disorders were postulated by Dameshek in the 1950s to be related to one another, proof came in 2005 when an acquired, mutation in the signaling enzyme Janus kinase 2 (Jak2V_{617}F) was identified in virtually all patients with PV, and ~50% of patients with ET and PMF[3]. The mutation was found to lead to constitutive activation of the kinase (actually, stabilization of the active form of the kinase), and to result in erythrocytosis and

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the pathognomonic finding in patients with MPNs, "spontaneous colony formation", in which marrow progenitor cells form blood cell colonies independent of hematopoietic growth factors. Since then a number of other mutations have been identified that account for the "Jak2V$_{617}$F negative" MPNs, including the thrombopoietin receptor, c-MPL, other mutations of Jak2, calreticulin and a variety of epigenetic modifier genes (e.g. TET2). Using a cell-specific Cre recombinase and SCT techniques we can introduce Jak2V$_{617}$F into murine megakaryocytes and platelets, hematopoietic stem cells, and endothelial cells, alone or in combination, in order to probe the role of the mutant kinase in various cells on several aspects of the MPNs. Using these tools we have found that the expression of Jak2V$_{617}$F in HSCs and ECs drives a MPN characterized by neutrophilia, thrombocytosis and splenomegaly, eventually evolving into myelosclerosis. Somewhat surprisingly, we found that Jak2V$_{617}$F-bearing ECs were required for many features of the MPN, such as enhancing the growth of Jak2V$_{617}$F-bearing HSCs over that of wild type HSCs, the radioresistance that characterizes MPN stem cells (as evidenced by relapse following SCT), and a hemostatic defect. Altogether, our studies suggest that the malignant vascular niche is a critical element in the pathogenesis of MPNs, and a more thorough understanding of the molecular basis for these findings could lead to improved treatment for patients with these disorders.

Results and Discussion

Megakaryocytes (MKs) are an important component of the perivascular stem cell niche.

As part of a study to generate a murine model of MPNs, we used an inducible human JAK2V$_{617}$F gene (termed FF1)[4] and drove its expression in HSCs and ECs using a Cre recombinase under the control of the Tie2 promoter ("Tie2/FF1 mice"). This model was chosen as much evidence indicates that mutant kinase is expressed in these cell types in patients with MPNs. As expected, these mice developed a robust MPN characterized by thrombocytosis, neutrophilic leukocytosis, splenomegaly and hematopoietic stem and progenitor cell expansion within 2 months of birth. As a control for these experiments, and to gauge the effects of JAK2V$_{617}$F-bearing MKs on MPN development, we also crossed FF1 mice with Pf4-Cre mice (which bear a Cre recombinase driven by the megakaryocyte-specific platelet factor 4 promoter)[4] to express JAK2V$_{617}$F exclusively in the MK lineage (Pf4^{+}FF1^{+}). Our expectation was that the Pf4^{+}FF1^{+} mice would develop thrombocytosis, splenomegaly, and greatly increased marrow megakaryopoiesis, but not a MPN. Somewhat surprisingly, the mice also developed significant increases in CD45^{+}EPCR^{+}CD48^{-}CD150^{+} (E-SLAM) cell numbers, a highly purified long-term repopulating HSPC population.[5,6] Rigorous efforts were undertaken to be certain that the mutant kinase was not also expressed in the HSC compartment in this mouse model, which it was not. Thus, the overabundance or qualitative changes (or both) of marrow MKs drove HSC expansion. Next, we found that MKs expressing JAK2V$_{617}$F overproduce chemokines and cytokines that favorably affect HSCs, including the cytokines fibroblast growth factor (FGF1) and stem cell factor (SCF), to help explain our findings. To determine whether the primary regulator of MK growth and development, thrombopoietin, or its receptor, c-Mpl were critical for this process, we crossed the Pf4^{+}FF1^{+} mice with Tpo$^{-/-}$ mice[7] or Mpl$^{-/-}$[8] mice to generate Tpo^{-}Pf4^{+}FF1^{+} mice and Mpl^{-}Pf4^{+}FF1^{+} mice. We found that neither the Tpo^{-}Pf4^{+}FF1^{+} mice nor the Mpl^{-}Pf4^{+}FF1^{+} mice developed any significant thrombocytosis, nor did they expand the numbers

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of E-SLAM cells despite the presence of the JAK2V_{617}F-bearing MKs in all of them. [9] Thus, these data and that of others indicates that the MK is a part of the hematopoietic niche and could play a role in the pathogenesis of MPNs.

The JAK2V617F-bearing vascular niche promotes JAK2V617F-mutant clonal expansion over JAK2WT clones both in vitro and in vivo.

To further explore the effects of the JAK2V_{617}F mutation on vascular niche function, we employed Tie2/FF1 mice, that express the mutant kinase in all hematopoietic cells (including HSCs) and ECs[10,11] as marrow transplant recipients of normal HSCs. Because the 950 cGy irradiation conditioning for transplantation should eliminate all HSCs and their progeny, the resultant chimeric mice should express JAK2V_{617}F only in ECs.

First, we used an *ex vivo* co-culture system in which wildtype or JAK2V_{617}F marrow lineage negative and cKit positive ($Lin^-/cKit^+$) cells, a population moderately enriched in HSCs, were cultured on a monolayer of wildtype or JAK2V_{617}F ECs (isolated from normal or $Tie2^+FF1^+$ mouse lungs). While there was no difference between wildtype and JAK2V_{617}F $Lin^-/cKit^+$ cell proliferation when co-cultured on wildtype ECs, compared to wildtype $Lin^-/cKit^+$ cells the JAK2V_{617}F hematopoietic cells displayed a relative growth advantage when co-cultured on JAK2V_{617}F ECs.[12]

Next, we performed competitive marrow transplantation experiments in which donor marrow cells from $Tie2^+/FF1^+$ mice were injected intravenously together with an equal number of wildtype marrow cells into lethally irradiated $Tie2^+/FF1^+$ mice or control mice. We used a CD45.1/CD45.2 genetic tracking system to assess the relative contributions of each donor mouse to recipient hematopoiesis during post-transplant recovery. During a 4-month follow up, $Tie2^+/FF1^+$ recipients displayed a greater level of peripheral blood JAK2V_{617}F-containing cell expansion than did control recipients. By 18 weeks post-transplant, $Tie2^+/FF1^+$ recipients of $Tie2^+/FF1^+$ and wildtype cells developed a profound MPN phenotype with neutrophilia, thrombocytosis, and moderate splenomegaly.[10] Quantitative evaluation of the marrow cells revealed significant increases in JAK2V_{617}F mutant-bearing $CD150^+CD48^-$ cells, a population of cells highly (~20%) enriched in HSCs in $Tie2^+/FF1^+$ recipients compared with control recipients. In contrast, control recipients of an equal mixture of $Tie2^+FF1^+$ and wildtype cells had mostly normal blood cell counts and there was no significant difference between the frequency of wildtype and mutant cells in the $CD150^+/CD48^-$ population. Therefore, the JAK2V_{617}F-bearing vascular niche promoted the expansion of JAK2V_{617}F HSCs at the expense of wildtype cells.[13]

The JAK2V_{617}F-bearing vascular niche protects JAK2V_{617}F HSCs from radiation injury both in vitro and in vivo.

To study the effects of the JAK2V_{617}F-bearing vascular niche on MPN hematopoiesis, we transplanted wildtype marrow cells directly into lethally irradiated (950cGy) $Tie2^+/FF1^+$ mice or age-matched littermate controls. During a 3-month follow up, while all wildtype control recipients displayed full donor engraftment, the majority of $Tie2^+/FF1^+$ recipient mice displayed partial recovery of JAK2V_{617}F-mutant hematopoiesis (mixed donor/recipient chimerism) 10 weeks following transplantation. The $Tie2^+/FF1^+$ recipients with mixed

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chimerism developed neutrophilia, thrombocytosis, splenomegaly, and mutant HSC expansion, similar to what have been observed in the primary $Tie2^+/FF1^+$ mice. [10,12] These results indicate that the $JAK2V_{617}F$-mutant HSCs in $Tie2^+/FF1^+$ mice are relatively protected from the otherwise lethal irradiation administered during conditioning for marrow transplantation.

Tie2-Cre mice express Cre recombinase in both ECs and hematopoietic cells. To investigate whether the radioprotection we observed was due to expression of the $JAK2V_{617}F$ mutation in $Tie2^+/FF1^+$ HSCs, we generated a chimeric murine model with $JAK2V_{617}F$-mutant HSCs and a wildtype vascular niche by transplanting $Tie2^+/FF1^+$ marrow cells into wildtype recipients. The transplantation of normal marrow cells into normal recipients served as a control. Following hematopoietic recovery and full donor cell engraftment, each set of mice were again irradiated with 300cGy to create a radiation injury, and 2 hour later marrow Lin^- cells were isolated and evaluated for cellular apoptosis. We found that in the wildtype vascular niche, compared to normal marrow, apoptosis was significantly *increased* (not decreased) in the $JAK2V_{617}F$-mutant containing cells. Thus, the HSC radioresistance seen in $Tie2^+/FF1^+$ mice was not due exclusively to expression of the mutant kinase in HSCs.

We next studied the effects of EC $JAK2V_{617}F$ expression on HSC radioprotection. Lin^- marrow cells were isolated from wildtype or $Tie2^+/FF1^+$ mice and cultured on primary EC feeder layers derived from wildtype or $Tie2^+/FF1^+$ murine lungs. The co-cultures were then irradiated with 300cGy and cells were counted and were assessed for viable hematopoietic progenitors (via colony formation) 24 hours after irradiation. Compared to being cultured on wildtype ECs, we observed higher total cell numbers and hematopoietic progenitors from $JAK2V_{617}F$ cells cultured on $JAK2V_{617}F$-bearing ECs, indicating that the $JAK2V_{617}F$-bearing vascular niche contributed directly to $JAK2V_{617}F$-mutant HSC radioprotection. To further explore this effect, we generated a chimeric murine model with wildtype HSCs and $JAK2V_{617}F$-bearing vascular niche by transplanting normal marrow cells into lethally irradiated $Tie2^+/FF1^+$ recipients. The transplantation of wildtype marrow cells into wildtype recipient mice served as a control. Six to ten weeks following transplantation, each set of mice were again irradiated with 300cGy to create a radiation injury, and 2 hour later marrow Lin^- cells were isolated and apoptosis assessed. We found that wildtype Lin^- cell apoptosis was decreased in mice with a $JAK2V_{617}F$-mutant vascular niche compared to a wildtype vascular niche.[14] Taken together, these data indicate that the $JAK2V_{617}F$-bearing vascular niche contributes directly to HSC radioprotection, which could be responsible for the high incidence of disease relapse in patients undergoing allogeneic stem cell transplantation for MPNs.[15–17]

The $JAK2V_{617}F$ mutation expands the vascular niche, potentially contributing to HSC expansion and radioprotection

We began to investigate how the $JAK2V_{617}F$ mutation alters vascular niche function to promote neoplastic hematopoiesis in MPNs. First, we found that the $JAK2V_{617}F$-bearing ECs (isolated from $Tie2^+FF1^+$ mouse lungs) displayed significantly increased cell proliferation, cell migration, tube formation and decreased radiation-induced apoptosis

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compared to wildtype ECs *in vitro*,[14] which could explain the increase in marrow vascularity seen in both Tie2^{+}/FF1^{+} mice,[11] and patients with MPNs.[18–20]

To help explain these findings we then measured the expression levels of CXCL12 and SCF, two essential niche factors important for HSC maintenance and survival,[21–23] in freshly isolated marrow ECs from control and Tie2^{+}/FF1^{+} mice. Both quantitative polymerase chain reaction (qPCR) analysis and flow cytometry analysis showed that compared to wildtype cells, CXCL12 and SCF were upregulated in JAK2V_{617}F-bearing marrow ECs. Moreover, the proportion of cells expressing the CXCL12 receptor CXCR4, or the SCF receptor c-Kit was significantly increased in the JAK2V_{617}F marrow cells compared to the corresponding wildtype cells. In to CXCL12 and SCF, epidermal growth factor[24] and pleiotrophin[25], two additional HSC stimuli, were also up regulated in irradiated JAK2V_{617}F ECs compared to wildtype ECs.[14] These results suggest that the JAK2V_{617}F-bearing vascular niche contributes to JAK2V_{617}F HSC expansion and radioprotection by its elaboration of HSC-active chemokines and cytokines.

The molecular mechanisms of niche function in normal and neoplastic hematopoiesis.

To begin to explore the molecular basis for the growth-promoting, radioprotection and other properties of the JAK2V_{617}F-bearing vascular niche we first tested the role of the Tpo/c-Mpl signaling system. Since its initial cloning and characterization it has been clear that this cytokine/receptor system is the primary regulator of megakaryocyte development and plays a non-redundant role in normal hematopoietic stem cell biology. More recently, work from our group and others demonstrated that c-Mpl is also essential for neoplastic HSC expansion and the development of a MPN in the Tie2^{+}/FF1^{+}-induced MPN.[26] c-Mpl is also expressed on several types of endothelium.[27–29] Whether the EC c-Mpl receptor contributes to the critical role of Tpo/c-Mpl signaling in HSC maintenance through an effect on the vascular niche is not known. We measured EC c-Mpl expression by qPCR and found that compared to wildtype cells, the receptor was upregulated in JAK2V_{617}F-bearing lung and marrow ECs,[12,13] suggesting that Tpo/c-Mpl signaling may affect both the general vasculature (e.g. lung) and the hematopoietic vascular niche (e.g. marrow) in JAK2V_{617}F-positive MPNs. We then tested whether Tpo affects EC function *in vitro*, and found that the hormone significantly stimulates EC cell migration in a dose-dependent fashion. In addition, the endothelial junction molecules ZO-1[30] and PECAM1 (platelet endothelial cell adhesion molecule, or CD31)[31] are upregulated in Tpo-treated ECs compared to untreated ECs, suggesting that Tpo/c-Mpl signaling may regulate vascular integrity, which is important for both hemostasis and tissue stem cell function.[30,32]

To begin to test the hypothesis that endothelial cell c-Mpl is important for the JAK2V_{617}F cell expansion in the hematopoietic vascular niche, we designed an *in vitro* competitive growth assay where both wildtype and JAK2V_{617}F Lin^{-}/cKit^{+} cells were cultured together (1:1 mix) in the presence of endothelial cell conditioned medium (ECCM) collected from either wildtype or c-Mpl$^{-/-}$ murine lung ECs. This type of experiment tests if ECs secrete a trophic factor for hematopoietic cells, and if genetic alteration of the ECs affects their functional niche effects. At the end of the 6-day culture, there were significantly more wildtype hematopoietic cells than those expressing JAK2V_{617}F in the presence of c-Mpl$^{-/-}$

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ECCM compared to wildtype ECCM, suggesting that the EC c-Mpl receptor is important for the maintenance/expansion of the JAK2V_{617}F cells over wildtype cells *in vitro*.[12] To determine the molecular basis for these findings, we tested chemokine and cytokine production in the ECs: we found that CXCL12 expression was significantly downregulated in c-$Mpl^{-/-}$ or $Tpo^{-/-}$ marrow ECs compared to control ECs. Since CXCL12 is important in directing MK migration toward the vascular niche and promoting MK maturation and platelet release,[33,34] the decreased CXCL12 expression in $Tpo^{-/-}$ and c-$Mpl^{-/-}$ marrow ECs could impair the interactions between MKs and ECs in the vascular niche. Consistent with this hypothesis, histological examination of the marrow revealed that MKs are less likely to be in direct contact with the sinusoidal vessels in the $Tpo^{-/-}$ and c-$Mpl^{-/-}$ mice compared to wildtype control mice, suggesting that Tpo/c-Mpl signaling can affect MK-EC interactions in the vascular niche.

The series of experiments discussed indicate that in addition to driving HSC expansion, the JAK2V_{617}F mutation also affects EC and MK contributions to the peri-vascular marrow niche in a model of MPN. One limitation of these studies is that every EC in both the *in vitro* and *in vivo* experiments reported express the mutant kinase. While there is little controversy over whether some ECs express JAK2V_{617}F in patients with MPNs, the proportion of mutant kinase expressing cells is uncertain. Hence, additional experiments need to be performed in which ECs chimeric for JAK2V_{617}F expression are present in vitro and in vivo. While the former experiments are straightforward, creating an *in vivo* model of EC chimerism is a technical challenge, but one that is currently under construction. It is hoped that these and other insights into the contributions of JAK2V_{617}F and Tpo/c-Mpl signaling to peri-vascular niche function in promoting MPN expansion and radio-resistance will contribute to better approaches to therapy of these disorders.

Additional molecular details of the mechanisms by which signals between the neoplastic and niche cells can provide a positive reinforcement loop are also being uncovered. For example, breast cancer cells can release lipid mediators of cancer signaling (sphingosine-1-P [S1P] and its receptor) that affect its metastatic niche[35], and since platelets (and likely, by extrapolation, megakaryocytes) can also release S1P, which can act on ECs, it is tempting to hypothesize additional mechanisms by which MKs might also affect the marrow niche[36]. These and other molecular mechanisms are under active investigation, so that a more complete understanding of the basis for marrow niche function in normal and neoplastic hematopoiesis will allow more effective therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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