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INVESTIGATION

Bone marrow fibrosis in primary myelofibrosis: pathogenic mechanisms and the role of TGF-β

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Abstract: Primary myelofibrosis (PMF) is a Philadelphia chromosome negative myeloproliferative neoplasm (MPN) with adverse prognosis and is associated with bone marrow fibrosis and extramedullary hematopoiesis. Even though the discovery of the Janus kinase 2 (JAK2), thrombopoietin receptor (MPL) and calreticulin (CALR) mutations have brought new insights into the complex pathogenesis of MPNs, the etiology of fibrosis is not well understood. Furthermore, since JAK2 inhibitors do not lead to reversal of fibrosis further understanding of the biology of fibrotic process is needed for future therapeutic discovery. Transforming growth factor beta (TGF-β) is implicated as an important cytokine in pathogenesis of bone marrow fibrosis. Various mouse models have been developed and have established the role of TGF-β in the pathogenesis of fibrosis. Understanding the molecular alterations that lead to TGF-β mediated effects on bone marrow microenvironment can uncover newer therapeutic targets against myelofibrosis. Inhibition of the TGF-β pathway in conjunction with other therapies might prove useful in the reversal of bone marrow fibrosis in PMF.

Keywords: Primary myelofibrosis (PMF); transforming growth factor beta 1 (TGF-β1); thrombopoietin (TPO); GATA-1

Introduction

Essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) constitute the classical group of BCR-ABL (Ph) negative chronic myeloproliferative disorders, now termed myeloproliferative neoplasms (MPN) (1). JAK2V617F is the best characterized mutation seen in these disorders with prevalence of more than 95% in PV, 50–70% in ET, and 40–50% in PMF (2). JAK2V617F mutation negative cases of PV can have JAK2 mutations in exon 12 present at a frequency of approximately 2–3% (3). JAK2V617F mutation negative cases of ET and PML can contain MPL mutations occurring at the frequency of 5%

to 10% (4). Recently, a mutation in the gene encoding CALR was found to occur in the majority of MPN patients with nonmutated JAK2 or MPL (5,6) (*Figure 1*). The mutations JAK2 and MPL kinases lead to constitutive stimulation of the JAK-STAT pathway leading to cytokine independent growth in PV and ET (7). Furthermore, there are a number of other genetic abnormalities being discovered like CSF3R and SETBP1 mutations in other MPNs like chronic neutrophilic leukemia indicating the multi-step pathogenesis and heterogeneity in this group of disorders (8,9). Even though these mutations can explain the pathogenesis of increased proliferation in MPNs, the pathogenesis of fibrosis in PMF is still very well elucidated.
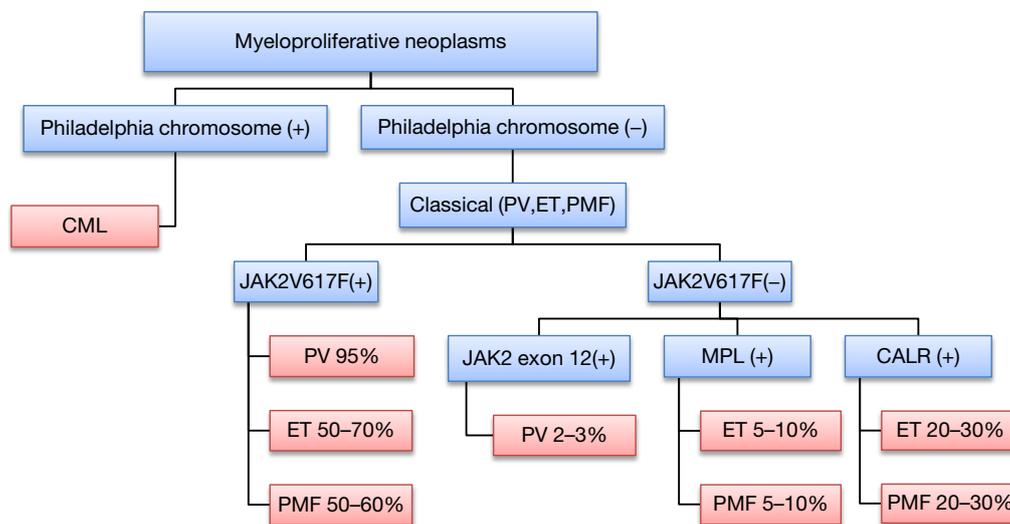
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Figure 1 Classification of myeloproliferative neoplasm. Others include chronic neutrophilic leukemia, chronic eosinophilic leukemia, hypereosinophilic syndrome, MPNs, unclassifiable. PV, polycythemia vera; ET, essential thrombocytosis; PMF, primary myelofibrosis; JAK2, Janus kinase 2; MPL, thrombopoietin receptor; CALR, calreticulin.

PMF carries the worst prognosis among the MPNs (10). The disease can start as PMF or present as the burnt out phase of PV (post-PV MF) or ET (post-ET MF) (11,12). It presents with anemia, splenomegaly and constitutional symptoms and is associated with a median survival of 6.5 years (10,11,13). Extramedullary hematopoiesis is seen not only in the liver and spleen but also in the lymph nodes, serosal surfaces, urogenital system and epidural and paraspinal spaces (12). Abnormal stem cell trafficking and extramedullary hematopoiesis occurs due to abnormalities in the CXCL12/CXCR4 axis (14). Bone marrow histology shows fibrosis, angiogenesis and osteosclerosis (15). Advanced reticulin or collagen fibrosis is associated with classic stages of PMF, but a diagnosis of PMF can be established without obvious fibrosis (15). Bone marrow fibrosis is the most important feature causing increased morbidity and mortality in these patients.

Pathogenesis of fibrosis in primary myelofibrosis (PMF)

The pathogenesis of MPNs is poorly understood (12). Even though mutations in JAK2, MPL and CALR are seen and can lead to constitutive action of the JAK-STAT pathway, it has not been elucidated how these mutations can cause different clinical phenotypes (16). In all MPNs, megakaryocytes proliferate, acquire multilobulated nuclei and exhibit clustering in the marrow (17). These megakaryocytes have abnormal location of P selectin on their intracytoplasmic vacuoles and demarcation membrane system (DMS) that leads to the increased emperipolesis (the passage of a cell into the cytoplasm of another cell) of neutrophils (18). The neutrophils release their enzymes in the megakaryocytes leading to the release of cytokines such as transforming growth factor beta (TGF-β); platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) from their alpha granules (18). These growth factors then stimulate fibroblasts to cause fibrosis and endothelial cells to cause neoangiogenesis (11). Increased production of osteoprotegerin by stromal and endothelial cells leads to unbalanced osteoblast proliferation, resulting in osteosclerosis and neoangiogenesis (19). Therefore, the disease is considered to be a clonal disorder of a hematopoietic/stem cell progenitor cell and the fibrosis that occurs is thought to be secondary due to cytokines released from the progenitor cells (20). *Figure 2* illustrated the proposed mechanism of fibrosis in myelofibrosis.

Bone marrow fibrosis is a secondary process

The prevailing hypothesis is that bone marrow fibrosis is a secondary process. It is believed that the mutant clone stimulates the production of fibrous tissue by fibroblasts that are polyclonal in nature as evident from cytogenetics and
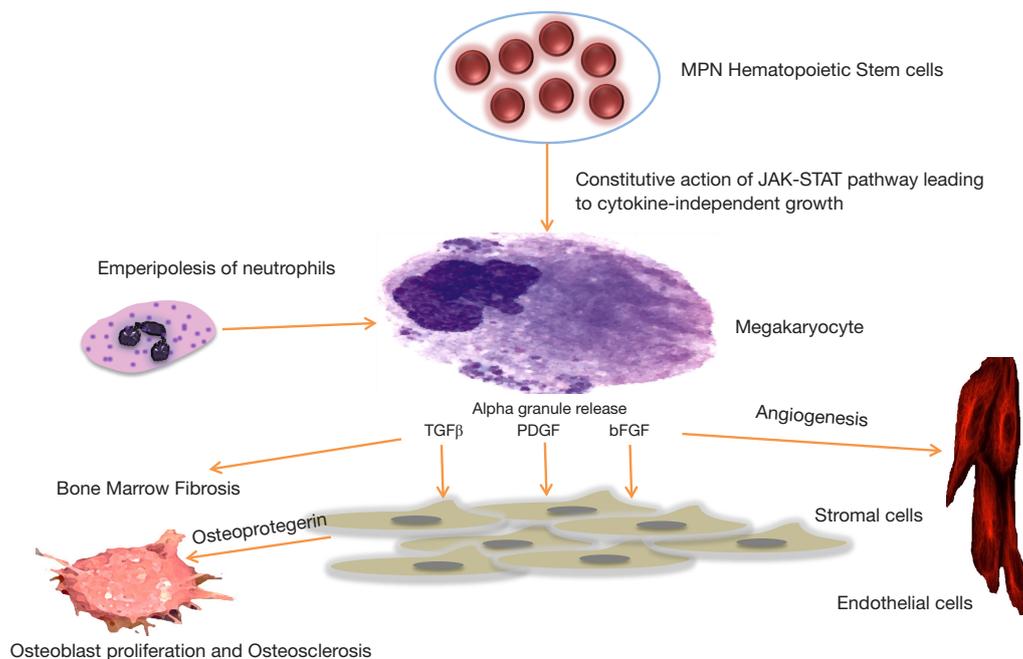
Figure 2 Pathogenesis of fibrosis: constitutive activation of JAK-STAT pathway in the MPN stem cell cohort leads to cytokine independent growth of megakaryocytes. Emperipolesis of neutrophils occurs that leads to release of TGF-β, PDGF and bFGF from alpha granules present in megakaryocytes. These cytokines cause fibrosis and angiogenesis and also lead to osteosclerosis by causing the stromal cells to release osteoprotegerin. MPN, myeloproliferative neoplasm; TGF-β, transforming growth factor beta; PDGF, platelet derived growth factor; FGF, basic fibroblast growth factor.

G-6PD expression analysis of these cells (20-22). The bone marrow fibroblasts have been shown to behave normally in culture (23) and do not carry the JAK2 mutation (24). Bone marrow transplantation is thought to be effective since it can remove the mutant clone and causes reversal of fibrosis (25). However, there have been recent data that challenges this concept. Some of the cells especially endothelial cells lining the hepatic sinusoids, micro vessels and large splenic veins of the spleen, have been shown to contain the JAK2 mutation (26,27). Endothelial progenitor cells have also been shown to be clonal (28). These data raise the possibility that some of the effector cells in marrow fibrosis might be related to the mutant clone.

A defective niche might contribute to the pathogenesis

The hematopoietic stem cells (HSCs) reside in the bone marrow microenvironment and have tightly regulated interactions with various cells in this niche (29). The bone marrow niche consist of fibroblasts, osteocytes,

and adipocytes derived from mesenchymal stem cells (MSCs), osteoclasts derived from hematopoietic cells and endothelial stem cells in addition to different growth factors and adhesion molecules (30). Two types of niches are described; the vascular niche and the endosteal niche (31). Whether or not there are two distinct niches or a combined hematopoietic environment is under debate, but there is evidence that there are distinct functional units. The vascular niche is located in the oxygen rich area close to the endosteum and regulates proliferation, differentiation and mobilization of the stem cells .The endosteal niche is located in the bone edge and keeps the stem cells in quiescence. An imbalance between the two niches has been suggested as a cause of myeloproliferative syndrome (31).

Two mouse models described by Walkley *et al*. gave us further insight into the importance of the microenvironment in causing myelofibrosis (32,33). Germ line deletion of retinoic acid receptor subtype-γ (RARγ) gene or the conditional knockout of the retinoblastoma (Rb) protein in the HSC compartment of the mice led to a myeloproliferative syndrome. Rb and RARγ are regulators of the cell cycle. In

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both mutant mice types, transplantation of the wild type HSCs did not lead to remission from the disease. When mutated HSCs were transplanted into wild-type mice, it did not cause myeloproliferation. Furthermore, only myeloid-restricted (compared to HSC) deletion of Rb also did not lead to myelofibrosis, hence highlighting the importance of microenvironment in the pathogenesis of this disease.

The hedgehog (Hh) signaling pathway has also been implicated in the pathogenesis of myelofibrosis using mouse models. Hh signaling pathway plays an important role in normal hematopoiesis and in the oncogenesis of hematologic malignancies. Inhibitors of the Hh pathway have been shown to inhibit growth and self-renewal capacity in preclinical models of MF. In a mouse model of MF, combined inhibition of the Hh and JAK pathways reduced JAK2 mutant allele burden, reduced bone marrow fibrosis, and reduced white blood cell and platelet counts (34).

Transforming growth factor beta (TGF-β) in bone marrow fibrosis

During hematopoiesis, TGF-β signaling pathway plays important roles in stem cell quiescence as well as in progenitor cell differentiation. This cytokine has been studied extensively as a regulator of fibrosis in myeloproliferative diseases (15). TGF-β occurs in 3 isoforms: TGFβ1, TGFβ2 and TGFβ3 (34). TGFβ1 is the most abundant of all these isoforms and platelets, megakaryocytes and bone marrow cells are sources of TGF-β production (35,36).

TGFβ1 is secreted as latent protein and is stored in the extracellular matrix. Latent complexes are classified as either small or large. The small complex consists of TGF-β noncovalently associated with a latency-associated protein (LAP). In the large complex, LAP is also linked to latent TGF-β-binding proteins (LTBPs) (37). Reactive oxygen species and a number of proteases including plasmin, integrins (38) and thrombospondin-1 (TSP-1) convert the inactive latent complexes to the active forms by releasing it from LTBP and LAP (37).

The active protein binds to two ubiquitous cell surface receptors—type I receptor (TBRI) and type II receptor (TBRII) with serine/threonine protein kinases in their intracellular domains (35). TGF-β binds to the TBRII which then recruits, transphosphorylates and binds to TBRI (31). Activated TBRI phosphorylates the Smad transcription factors (R-Smads 2/3) which then bind to a common Smad 4 protein (15,35,37). R-Smad/Co-Smad complexes translocate to the nucleus, where they associate with DNA-binding partners and then regulate the transcriptional response of the TGF-β target genes (35,39,40) (*Figure 3*). Regulation of the TGF-β pathway is accomplished by multiple negative feedback mechanisms including: down-regulating expression of cell surface receptors, increasing inhibitory Smad 6 and 7 which repress TGF-β responses, activating latent proteins, and initiating interaction of multiple transcriptional co-repressors and co-activators inside the nucleus (35).

Role of TGF-β in bone marrow fibrosis in Philadelphia negative myeloproliferative disorders

Bone marrow pathology in PMF is characterized by fibrosis, neoangiogenesis and osteosclerosis (15). Fibrosis is primarily due to an increase in production of total collagen, including types I, III, IV, and V collagens (41). Increased deposition of laminin and adhesive glycoproteins (vitronectin, fibronectin and tenascin) is seen in advanced stages of the disease. TGFβ1 increases the synthesis of types I, III and Type IV collagen in addition to the deposition of fibronectin, proteoglycans and tenascin (42). The fibrotic process induced by TGFβ1 is the combination of an increase in matrix biosynthesis, accompanied by a decrease in matrix degradation. TGFβ1 does this by decreasing the amount of matrix metalloproteinases (MMP) particularly MMP3 and increasing the synthesis of tissue inhibitors of metalloproteinase (TIMP) particularly TIMP-1 (43).

In addition to effects on the microenvironment, TGF-β has direct effects on hematopoietic cells also and is a well-known negative regulator of granulocyte, erythroid, megakaryocyte and macrophage progenitor proliferation and promotes their differentiation (44). It has been seen that hematopoietic cells in Philadelphia chromosome negative myeloproliferative disorders frequently become resistant to the effects of TGF-β due to decreased levels of TGF-β receptors and regulators. ET, PV and PMF have been associated with decreased levels of type II receptors while decreased levels of SMAD4 have been seen in some cases of ET. These data suggest that effects of TGF-β may be dominant on the bone marrow microenvironment (45,46).

Evidence from mouse models of the disease

Mouse models of myelofibrosis have provided important insights into the role of TGF-β in the pathogenesis of fibrosis (47-55). Two widely studied mouse models include
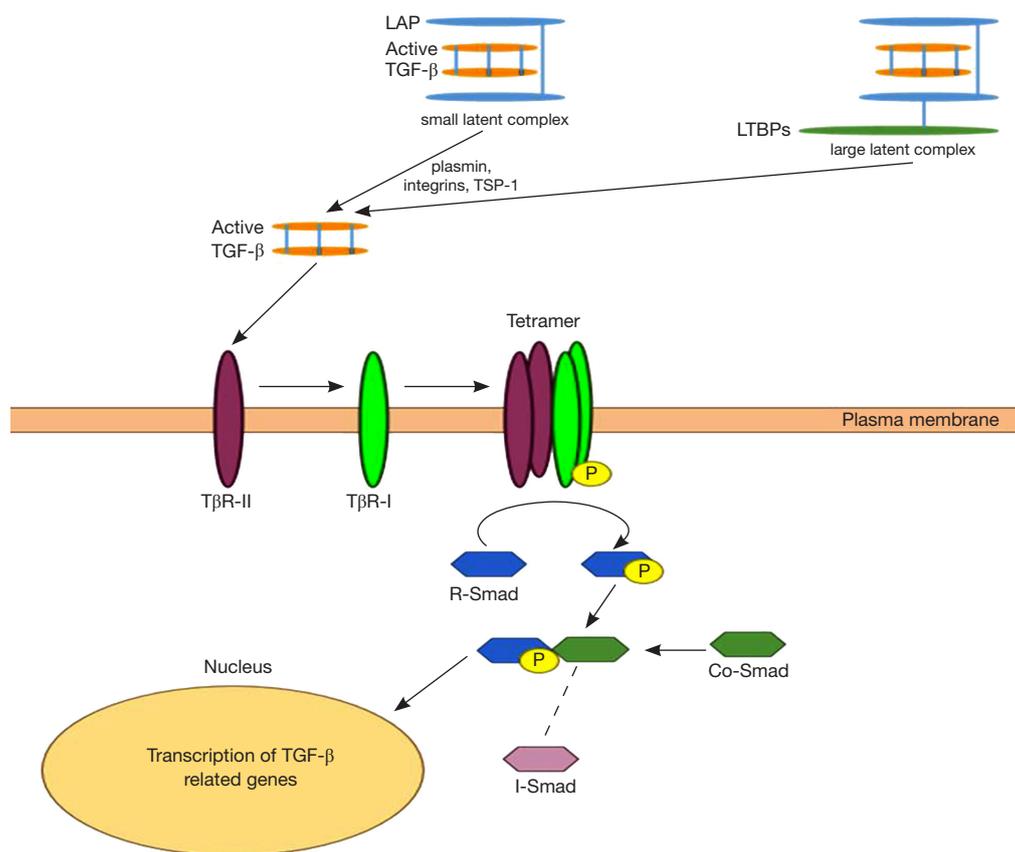
Figure 3 Transforming growth factor beta signaling pathway TGFβ1 latent proteins are present in 2 forms; a small complex consists of TGF-β noncovalently associated with a latency-associated protein (LAP) and a large latent complex in which LAP is linked to latent TGF-β-binding proteins (LTBPs). Integrins, plasmin and thrombospondin 1 (TSP-1) convert the inactive latent complex to the active form by releasing it from LTBP and LAP. The active TGF-β I binds to the type II receptor (TRII) which then recruits, transphosphorylates and binds the type I receptor (TRI). The activated TRI phosphorylates the transcription factors R-Smads (Smad 2/3) which then bind to the common Smad 4. R-Smad/Co-Smad complexes translocate to the nucleus, where they associate with DNA-binding partners and then regulate the transcriptional response of the TGF- β target genes. Inhibitory Smad 6 and 7 repress TGF-β responses.

the thrombopoietin (TPO) high and the GATA1 low mice. High doses of TPO result in megakaryocyte abnormalities similar to PMF patients and lead to a myeloproliferative syndrome with deposition of reticulin fibers in the bone marrow (47-49). Consequently, mice exposed to high doses of TPO transfected via a retrovirus vector developed a myeloproliferative syndrome with proliferation of megakaryocytes, extramedullary hematopoiesis, splenic and medullary fibrosis and osteosclerosis similar to PMF in humans (49,50). TGF-β in these mice models was significantly increased in the extracellular fluid of the marrow, plasma and platelet extracts (49). To determine the role of TGF-beta in this process, bone marrow stem cells obtained from knockout models of TGF-β1 gene

(TGF-β1$^{-/-}$) and wild-type (WT) mice were infected with a retrovirus encoding the murine TPO protein. These marrow cells were then engrafted into lethally irradiated wild-type hosts for long-term reconstitution. Both groups of animals developed a myeloproliferative syndrome with thrombocytosis, leukocytosis, splenomegaly, extramedullary hematopoiesis and increased progenitors in the blood. However the wild type mice developed severe reticulin fibrosis in the bone marrow and spleen compared to the null (TGF-β1$^{-/-}$) mice and had latent TGF-β1 levels in the plasma and spleen that were 4 and 6 fold higher. These data demonstrate the important role of TGF-β as an effector of fibrosis in PMF (51).

Another mouse model that has been utilized to study

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myelofibrosis and myeloproliferation is the GATA-1 low mice. GATA-1 is a transcription factor that has a well-established role in erythroid and megakaryocytic cell differentiation (52). Absence of GATA-1 can also lead to myelofibrosis which was proved by the GATA-1 low mouse model. There was delay in the development of myelofibrosis in the GATA-1 low mice, starting at 15 months of age (55) but GATA-1 low mice eventually developed bone marrow fibrosis, presumably from a block in the maturation of megakaryocytes into proplatelets, resulting in accumulation of megakaryocytes in the marrow and spleen in the presence of lower GATA-1 levels compared to WT littermate (53,55). TPO exposure as discussed above is a contributing factor in developing reticulin fibrosis in WT mice models. In contrast, GATA-1 low mice treated with TPO do not develop liver and spleen fibrosis and TPO treatment leads to restoration of GATA-1 level in the megakaryocytes and down regulation of the TGF-β gene (47). A recent paper studied the role of TGF-β in the bone marrow and spleen of GATA-1 low mice (54). Alterations of TGF-β1, Hh, and p53 signaling in marrow and spleen and of mammalian target of rapamycin (mTOR) in spleen only were identified in GATA-1 low mice. Inhibition of TGF-β1 signaling in these GATA-1 low mice by an inhibitor of the tyrosine kinase activity of TGF-β1 receptor type I, led to normal development of megakaryocytes, and these mice had reduced fibrosis, neovascularization, and osteogenesis in the bone marrow, thus reducing extramedullary hematopoiesis in the spleen (54).

Evidence from human studies

Involvement of TGF-β in myelofibrosis was first examined in patients with acute megakaryoblastic leukemia with bone marrow fibrosis. Megakaryocytes produce and secrete an active form of TGF-β which was able to stimulate collagen synthesis by myofibroblasts *in vitro* (56). Intraplatelet levels of TGF-β were found to be elevated 2–3 fold in PMF patients as compared to controls (57). In a trial of six subjects, interferon-γ therapy for 6 months reduced the intraplatelet levels of TGF-β to almost normal in 4 subjects (58). Increased TGF-β levels were also found to be elevated in peripheral blood mononuclear cells both at the mRNA and peptide level (59). While TGF-β levels in CD34+ cells with myelofibrosis were not elevated, the expression of TGF-β II receptor was significantly decreased. TGF-β has a negative regulatory effect on CD34+ hematopoietic progenitor cells, thus in decreasing the TGF-β type II receptor, the CD34+

cells escape regulatory controls (60). Hypermethylation of the promoter of the TGF-β II receptor appears to cause the decrease in the level of the receptor (61).

In this complex disease, elevation of TGF-β alone is not the only cytokine responsible for producing fibrosis seen in myelofibrosis. Numerous studies indicate that other cytokines such as substance P (62), bFGF (63), vascular endothelial growth factor VEGF (64) , increase in TIMPS and decrease in MMP (43) are also likely to be important in the pathogenesis of bone marrow fibrosis , illustrating the complexity of this disease and its treatment.

Targeting TGF-β in myelofibrosis

The discovery of the JAK2 mutation and subsequently the development of new JAK2 inhibitors have been instrumental landmarks in the treatment of symptoms in MF (65). However, unlike the tyrosine kinase inhibitors for CML, the JAK2 inhibitors have been unable to eradicate the mutant clone (66). JAK2 inhibitors have been successful in reducing spleen size and decreasing constitutional symptoms, but have not been able to reduce bone marrow fibrosis (67).

TGF-β inhibition is a potential therapeutic strategy to decrease marrow fibrosis in MPNs. Inhibition of TGF-β is also being investigated in other clinical and experimental scenarios. The levels of active TGF-β can be decreased by targeting the conversion of the latent form to the active form. Integrin αvβ6 plays an important role in converting the inactive latent complex to the active form by releasing it from LTBP and LAP as described above. Monoclonal antibody that blocks α(v)β6 integrin was evaluated in murine model of pulmonary fibrosis and it showed reduction in pulmonary fibrosis (68). A number of neutralizing antibodies have also been used against TGF-β, for example Fresolimumab (GC1008) that targets all three TGF-β isoforms. This was used in a phase I trial of treatment resistant primary focal segmental glomerulosclerosis (69) and myelofibrosis (70). Another method is to prevent the binding of TGF-β to cell surface receptors. This was demonstrated by topical use of P144, a peptide inhibitor of TGF-β1, that ameliorated skin fibrosis in a well-characterized murine model of human scleroderma (71).

The intracellular effects of TGF-β can be blocked by preventing receptor kinase activity. A small-molecule kinase inhibitor that blocks activin-like receptor kinase 5, SM305, was used in an experimental study. It showed that it blocked receptor kinase SMAD dependent activation of fibroblasts both in cell culture and mice (72).

Recent development of inhibitors of TGF-β receptor I kinase is very promising. A new molecule Galunisertib (LY2157299) is a specific inhibitor of TGF-β receptor I kinase and abrogates the phosphorylation of SMAD2. This was studied in a mouse model generated by transgenic expression of JAK2V617F that display PV-like at the age of 6 weeks and develop bone marrow and spleen fibrosis at 25–30 weeks. When these mice with established myelofibrosis were treated with LY2157299 daily for 4 weeks, a significant decrease in fibrosis was seen. This was also validated in MPLW515L transplantation mouse model with myelofibrosis, hence proving the concept of reversal of myelofibrosis with TGF-β inhibition (73).

Gene transfer of negative regulator SMAD 7 has been used in experimental renal fibrosis in mice (74). Non SMAD pathways like c-Abl that leads to TGF-β mediated fibrotic response have also been targeted and the use of Imatinib (Abl kinase inhibitor) for this purpose is a classic example (75).

Another way of targeting TGF-β is by inhibition of Aurora kinase. Megakaryocytes in PMF show impaired differentiation and Aurora kinase inhibition induces megakaryocyte maturation, thereby reducing the burden of immature megakaryocytes. This ameliorates the characteristics of PMF, including bone marrow fibrosis. This concept was proven in murine model by treatment with MLN8237, a selective AURKA inhibitor. MLN8237 promoted polyploidization and differentiation of megakaryocytes with PMF-associated mutations and had potent antifibrotic and antitumor activity *in vivo* in mouse models of PMF (76).

These studies have shown very encouraging results so far. Our hope is to have a greater comprehension of development of fibrosis in MPNs, leading to an era of exciting therapeutic development in this very difficult to treat disease.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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