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Review article

Clinical applications of thrombopoietin silencing: A possible therapeutic role in COVID-19?

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ABSTRACT

Thrombopoietin (TPO) is most recognized for its function as the primary regulator of megakaryocyte (MK) expansion and differentiation. MKs, in turn, are best known for their role in platelet production. Research indicates that MKs and platelets play an extensive role in the pathologic thrombosis at sites of high inflammation. TPO, therefore, is a key mediator of thromboinflammation. Silencing of TPO has been shown to decrease platelets levels and rates of pathologic thrombosis in patients with various inflammatory disorders (Barrett et al, 2020; Bunting et al, 1997; Desai et al, 2018; Kaser et al, 2001; Shirai et al, 2019). Given the high rates of thromboinflammmation in the novel coronavirus 2019 (COVID-19), as well as the well-documented aberrant MK activity in affected patients, TPO silencing offers a potential therapeutic modality in the treatment of COVID-19 and other pathologies associated with thromboinflammation. The current review explores the current clinical applications of TPO silencing and offers insight into a potential role in the treatment of COVID-19.

1. Introduction

Thrombopoietin (TPO) is a polypeptide consisting of 353 amino acids including a 21 amino acid secretory leader sequence [1]. The amino-terminal end binds to the TPO receptor, c-Mpl. TPO is most recognized for its function as the primary regulator of megakaryocyte (MK) expansion and differentiation. TPO is able to facilitate proliferation of MK progenitor cells, increase the number and size of MKs, and support MK maturation [2]. MKs, in turn, are the primary producers of platelets, which play a key role in hemostasis.

In addition to its importance to MKs, TPO also plays a role in the development of hematopoietic stem cell and progenitor cell populations. *In vitro* studies have demonstrated that TPO facilitates proliferation of hematopoietic progenitor cells, especially in combination with interleukin-3 (IL-3) or stem cell factor (SCF) [3]. In addition, TPO facilitates hematopoietic stem cell expansion, as evidenced by congenital forms of amegakaryocytic thrombocytopenia developing as a result of c-Mpl mutations in humans. In affected children, aplastic anemia develops within the first 5 years of life due to a diminishing number of hematopoietic progenitor cells [4].

c-Mpl is a member of the type I cytokine receptor family. Similar to other type I cytokine receptors, c-Mpl recruits Janus kinase (JAK) prior to the receptor integrating within the cell membrane. After TPO binding to c-Mpl, JAK2 becomes activated and subsequently initiates STAT3 and STAT5, which then induce transcription within the nucleus [5]. At the same time, TPO activates suppressors of cytokine signaling that work to degrade the activated c-Mpl receptor, thereby autoregulating TPO signaling [1].

Northern blot analyses of multiple organs reveal that TPO is widely expressed with mRNA being present at highest levels in the liver, but also found within the kidneys, smooth muscles, and bone marrow stromal cells [1,6]. Under normal physiologic conditions, plasma concentrations of TPO vary inversely with platelet count. Platelets have the ability to absorb constitutively synthesized TPO and destroy it, thereby

Abbreviations: TPO, Thrombopoietin; MK, Megakaryocyte; IL-3, Interleukin-3; SCF, Stem cell factor; JAK, Janus kinase; VEGF, Vascular endothelial growth factor; CRS, Cytokine release syndrome; COVID-19, Coronavirus disease 2019; TPO-ASO, Thrombopoietin gene antisense oligonucleotides; MMTV-PyMT, Mouse mammary tumor virus-polyoma middle tumor-antigen; DIC, Disseminated intravascular coagulopathy; NF-E2, Nuclear factor erythroid 2; GalNAc, N-acetylgalactosamine; IFN, Interferon; NETs, Neutrophil extracellular traps.

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helping to maintain a physiologic balance of the cytokine.

Despite multiple mechanisms whereby TPO is autoregulated, many inflammatory states are associated with TPO levels well above expected levels based on patient platelet counts. Indeed, significant elevation of TPO and secondary thrombocytosis is observed in many clinical inflammatory conditions such as myeloproliferative disorders, malignancy, and autoimmune disorders. This elevation in TPO and platelet count is mediated by IL-6, which increases TPO production both *in vitro* and *in vivo* [1]. In fact, IL-6 knockout mice do not demonstrate increases in TPO levels during inflammation [7]. Further, platelet levels in these mice are lower during inflammatory conditions compared to wild-type mice. This is directly related to a lack of an IL-6-mediated increase of mRNA transcription of TPO by the liver during periods of inflammation [7].

IL-6 plays an important role in inflammatory and neoplastic disease. IL-6 is among the most potent regulators of acute inflammation. Administration of IL-6 has been demonstrated to increase acute phase inflammatory reactants and platelet count. In addition, overexpression has been found in most types of tumors [8]. Further, IL-6 has been found to be significantly higher in patients with reactive thrombocytosis than in healthy controls [9].

Excessive IL-6 signaling contributes to end organ damage by maturing naïve T cells into effectors, inducing vascular endothelial growth factor (VEGF) expression in epithelial cells, and increasing vessel permeability. Cytokine release syndrome (CRS) refers to an uncontrolled and overwhelming release of proinflammatory mediators, including IL-6, by an overly activated immune system [10]. Thromboinflammation, defined as the convergence of thrombotic and inflammatory processes, is well recognized in the context of CRS [11]. Thromboinflammation is a pathologic condition described extensively in the context of sepsis, ischemia-reperfusion injury, major trauma, and several other disease entities [11]. Central to thromboinflammation is the loss of normal antithrombotic and anti-inflammatory function of endothelial cells, leading to dysregulation of coagulation, complement, platelet activation, and leukocyte recruitment in the microvasculature [12]. When severe, thromboinflammation can extend systemically and damage remote organs, particularly the lung and kidneys. This may lead to development of multiorgan dysfunction and death [11]. Despite use of anticoagulation therapies in the past, bleeding complications undermine clinical benefit. Thus, defining molecular mechanisms regulating thromboinflammation in specific disease states is of major clinical importance.

Given the relationship between CRS, thromboinflammation, TPO, MKs, and platelets, TPO silencing offers a potential therapeutic modality for the numerous pathologies in which thromboinflammation is a defining feature of the disease. Indeed, in patients suffering from severe illness due to the novel coronavirus disease 2019 (COVID-19), thromboinflammation and pathologic MK and platelet manifestations are becoming increasingly recognized hallmarks of the disease [13,14]. In the current review, we aim to describe the current clinical context for TPO silencing and discuss a possible mechanism for its use in patients with thrombocytosis associated with myeloproliferative disorders, malignancy, autoimmune disorders, and COVID-19.

2. Clinical applications of TPO silencing

2.1. Myeloproliferative neoplasms

Myeloproliferative neoplasms are a group of disorders in which the bone marrow makes too many abnormal mature blood cells of the myeloid lineage [15]. These disorders uniformly originate within pluripotent hematopoietic stem and progenitor cells as a result of mutations that provide selective advantage and promote differentiation to a specific myeloid phenotype [16]. Myeloproliferative neoplasms are associated with constitutive activation of JAK2 downstream of the c-Mpl receptor. Over 90% of myeloproliferative neoplasms have mutations in genes that encode JAK2, calrecticulin, or c-Mpl [16]. c-Mpl mutations induce the MK-related myeloproliferative neoplasms, essential thrombocythemia, and primary myelofibrosis, due to the fact that c-Mpl is selectively expressed on hematopoietic stem cells destined to differentiate along the MK pathway [17]. Current therapies for myeloproliferative neoplasms focus on inhibition of the JAK2 pathway [15]. However, JAK2 inhibitors do not preferentially target myeloproliferative initiating cells and therefore rarely induce remissions [17].

TPO metabolism is significantly altered in myeloproliferative neoplasms [18]. Loss of the TPO receptor, c-Mpl, expression on MKs and platelets results in loss of TPO clearance and higher TPO production, which, in turn, increases JAK2 signaling. Previous studies have demonstrated that mutations within the c-Mpl pathway in patients with myeloproliferative neoplasms may be hypersensitive to TPO, resulting in skewed signaling [19]. Further, overexpression of TPO in mice leads to the development of lethal myeloproliferative disorders or myelofibrosis [20]. Recent data have also demonstrated a reduction in hematopoietic stem cells in the absence of TPO genes, supporting the hypothesis that myeloproliferative neoplasms are growth factor-dependent diseases [18]. Given these facts, TPO silencing may offer a potential therapeutic target for patients with myeloproliferative neoplasms [18]. In addition, thrombosis and thrombocytosis are both predictive of poor survival in patients with myeloproliferative neoplasms and many treatment goals for patients are aimed at prevention of these complications [21]. Since TPO induces thrombocytosis and subsequent thrombosis, TPO silencing offers the additional advantage of lowering platelet counts and thrombotic complications.

2.2. Cancer

TPO silencing has been utilized in many animal models of cancer. The cancer-related synthesis of IL-6 and subsequent increase in TPO signaling may explain the high levels of platelets found in cancer patients [22]. Importantly, high platelet count is a negative prognostic factor in many cancers [23]. The *c-Mpl* proto-oncogene is expressed in a sizable portion of blast cells from patients with myeloid malignancies, and its expression appears to adversely affect survival in patients with acute myeloid leukemia or myelodysplastic syndromes [24].

Shirai et al. [25] synthesized murine- and primate-specific hepatic TPO gene antisense oligonucleotides (TPO-ASO) that silence hepatic TPO expression without blocking extrahepatic TPO. TPO-ASO was administered to 6-week-old transgenic mouse mammary tumor virus-polyoma middle tumor-antigen (MMTV-PyMT) mice that develop early ductal atypia that progresses into c-Mpl-negative fatal metastatic breast cancer within 2–3 months. TPO-ASO treatment significantly increased average time to 2-cm^3 palpable tumor volume and decreased tumor proliferation index, tumor vascularization, and pulmonary metastases compared to controls.

In addition to the role TPO and c-Mpl play in cancer, platelets have been implicated in neoplastic proliferation. Cancer patients frequently present with signs of thrombosis including disseminated intravascular coagulopathy (DIC), migratory thrombophlebitis, and pulmonary embolism – all signs of aberrant platelet activation and aggregation.

Tumor cells are theorized to enter the blood stream and bind to and activate platelets and leukocytes. These platelet/tumor aggregates then adhere to the vessel wall and escape immune surveillance. This allows for both local tumor survival as well as a means for metastatic spread through the vasculature [23].

Along with platelets providing immune protection to tumor cells, VEGF is released by platelets upon their activation which can promote angiogenesis [26]. Moreover, platelets can change the permeability of the vasculature. Among other factors, platelets release serotonin which contributes to changes in vascular tone and histamine which increases vascular permeability [23,27]. The leaky tumor vasculature may expose matrix proteins that potently activate platelets, leading to further angiogenesis [23]. In experimental models, blockage of serotonin

receptors hinders metastasis [28]. Further, genetic reduction of platelets via knocking out the nuclear factor erythroid 2 (NF-E2) gene that contributes to MK maturation have been found to decrease metastases in mice models [29]. In fact, experimental metastases are nearly completely inhibited in these mice. Thus, TPO silencing may allow for safe platelet reduction in cancer patients, which may improve outcomes [30].

2.3. Autoimmune disorders

A growing body of evidence indicates that some autoimmune disorders such as rheumatoid arthritis and systemic lupus erythematosus are regulated by components of the hemostatic system [11]. Rheumatoid arthritis is characterized by local joint bone erosion and systemic bone loss. Serum IL-6 levels are uniformly elevated in patients with rheumatoid arthritis as IL-6 is the main cytokine driving inflammation in these patients [31]. Further, IL-6 is the most abundantly expressed cytokine in the synovial fluid of patients with rheumatoid arthritis. IL-6 enhances bone resorption in patients with rheumatoid arthritis, causing a net overall loss of bone density in these patients [32].

As previously discussed, IL-6 increases TPO levels. TPO signaling then results in eventual shedding of platelets by mature MKs [32]. Upon activation, platelets shed small vesicles called microparticles that are abundant in the synovial fluid of patients with inflammatory arthritis. These microparticles release IL-1, which exacerbates inflammation. In addition, platelets promote vascular permeability in the arthritic joint by serotonin release. Platelets also produce prostaglandin 12, which is another key factor in inflammatory arthritis. Furthermore, thrombocytosis correlates with disease severity in rheumatoid arthritis [32]. TPO silencing may therefore be utilized to decrease symptoms of inflammatory arthritis through platelet depletion. Notably, platelet depletion has been found to protect against lupus in mouse models [33].

2.4. Platelet depletion

As previously discussed, the main regulator of platelet count is TPO. At high levels, platelets support pathological thrombosis. Multiple previous animal studies have demonstrated the efficacy and safety of TPO silencing as a means to decreasing platelet numbers safely. TPO inhibition has been found to be safe with regards to the moderate reduction in platelet count in primates without adverse bleeding consequences [30]. Further, global TPO knockout mice have a 90% reduction in MK and platelet level. They also do not exhibit excessive bleeding [34]. MKs and platelets in these mice are morphologically and functionally normal, and importantly these mice survive into adulthood [34]. To date, the only documented adverse event associated with TPO silencing in TPO silencing models is prolonged bleeding time when platelet levels are allowed to drop to levels less than 100,000/ μL [25,30,34]. Notably, this is the threshold for increased bleeding time in any cause of thrombocytopenia [30]. Another advantage of TPO silencing for platelet reduction is that it allows for normal platelet function such that in cases of severe thrombocytopenia new platelets may be reintroduced without adverse effects on their function. This is in contrast to other anti-platelet medications such as aspirin and clopidogrel that directly impair platelet function and remain in circulation for several days after administration [30]. In addition, TPO may be directly administered to patients quickly increase TPO levels to a physiologic range.

Barrett et al. [35] described generating TPO-ASO to reduce circulating platelet count. The authors found that TPO silencing reduces platelet, MK, and MK progenitor counts without altering platelet activity. Further, this was achieved acutely and sustained chronically without adverse bleeding consequences. Similarly, Desai et al. [36] developed a trivalent N-acetylgalactosamine (GalNAc)-siRNA to silence TPO gene expression in the liver of mice. TPO liver mRNA levels were reduced by up to 80% after a single TPO siRNA dose, with no effect on TPO mRNA expression in other organs. Circulating TPO levels were reduced by 80% by day 7 and were suppressed for up to 28 days after a single treatment. Platelet counts were reduced to 60% by day 14 and 70% by day 21. The platelet reduction was accompanied by a 50% decrease in bone marrow MKs compared to controls.

In the aforementioned study by Shirai et al. [25] the authors synthesized murine- and primate-specific TPO-ASO that silence hepatic TPO expression without blocking extrahepatic TPO. Repeated doses of TPO-ASO were administered to mice and a baboon, causing a sustained 50% decrease in plasma TPO levels and platelet count within 4 weeks in both species. In addition to the previously mentioned effects, TPO-ASO reduced plasma platelet factor 4, VEGF, bone marrow MK density, and macrophage count. This was maintained in a safe hemostatic platelet count range [25].

3. Possible therapeutic role of TPO silencing in COVID-19

On March 11, 2020, the World Health Organization declared COVID-19 a global pandemic. As of March 12, 2021, COVID-19 had resulted in a total of 118,719,900 confirmed cases and 2,632,147 global deaths [37]. Although respiratory compromise is the cardinal feature of the disease, early studies have suggested that elevated platelet levels are associated with mortality in COVID-19 [13]. In addition, patients also demonstrate a high rate of thromboembolism [12]. Elevated inflammatory markers, including C reactive protein and erythrocyte sedimentation rate, were also associated with thrombosis and mortality [12].

COVID-19 series report hyperinflammation governed by pro-inflammatory cytokines in particular in patients with poor outcome, indicating a significant role of cytokine release for tissue damage and multiorgan failure [38]. Data have demonstrated that acute respiratory distress syndrome occurs in some SARS patients despite a diminishing viral load, suggesting an exuberant host immune response rather than viral virulence is the cause for tissue pathologies. Therefore, antiviral therapy alone may be inadequate [10].

Recent autopsy reports have shown that in patients who died from COVID-19, there were 3-fold higher than normal numbers of MKs in several organs, including the lungs and heart [14,39]. In addition, MKs have been found in cortical capillaries throughout the brain in patients who died from complications of COVID-19 [40]. In fact, these cases were the first report of MKs found in cerebral capillaries for any cause in the medical literature. Further, platelet-rich thrombi were found extensively at multiple end-organ sites. Thus, it appears that many patients with COVID-19 have significant pathologic MK and platelet manifestations. Given the high rate of clinically significant thrombus formation in patients who have died from COVID-19, a mechanism whereby platelet-rich thrombi can be decreased, and clinical outcomes improved, is warranted.

The pathogenesis of CRS in COVID-19 is incompletely understood. It is believed that the delayed kinetics of virus clearance is the trigger [10]. After initial host immune system evasion and subsequent viral replication, SARS-CoV evokes plasmacytoid dendritic cells and macrophages, launching a delayed but robust type I interferon (IFN) response and releasing other inflammatory cytokines against SARS-CoV [10]. Consequently, the activation of type I IFN signaling attracts inflammatory cells to the lung. This process amplifies the innate response, forming a viscous pro-inflammatory cycle. The overwhelming cytokines and chemokines cause localized pulmonary injury characterized by diffuse alveolar damage and dysregulated coagulation.

While IL-6 is uniformly elevated in COVID-19 patients with severe forms of the disease, Sanofi and Regeneron discontinued phase 3 clinical trials with human monoclonal antibody to the IL-6 receptor based on failure to reduce the need for ventilation or improve survival in COVID-19 patients [13]. Several other clinical trials are also looking at the use of JAK-STAT inhibitors. However, care must be taken as several of these drugs have black-box warnings for thromboembolism, which as discussed above, is a very common complication in COVID-19 patients [41]. In contrast, previous studies have demonstrated that TPO

inhibition neutralizes IL-6-induced thrombocytosis and protects against thromboembolism, which highlights a possible therapeutic role of TPO silencing in COVID-19 patients [22].

3.1. Thrombosis

Platelet activation underlies thrombus formation. A multicenter retrospective study demonstrated that platelet count $> 450 \times 10^9$/L was predictive of thrombosis in COVID-19 patients. As such, the elevated MKs and platelets are likely contributing to the hypercoagulable states associated with COVID-19 patients [13]. Recent autopsy reports documented up to a 3-fold increase of MKs in the lungs and hearts of COVID-19 patients who died from the disease [14]. In addition, these reports demonstrated a remarkably high number of platelet-rich thrombi in multiple end-organ systems. Alveolar capillaries were filled with fibrin-rich thrombi containing neutrophils, indicating extensive platelet activation. These thrombi were also directly associated with myocardial infarction unrelated to underlying atherosclerosis as well as acute tubular necrosis in the absence of renal glomerular pathology. Notably, these pathologies were found in the absence of other causes of hypercoagulable states such as DIC or superimposed secondary infection [14]. Elevated levels of platelet/ fibrin thrombi were also noted in COVID-19 patients on pharmacologic anticoagulation treatments, suggesting investigation of alternative therapies, such as TPO silencing, aimed at decreasing COVID-19 related hypercoagulable states is warranted. As mentioned previously, TPO silencing has been safely used to reduce platelet count and rates of thrombosis formation.

3.2. Pulmonary disease

The primary cause of death in COVID-19 appears to be acute lung injury characterized by severe endothelial damage, inflammation, and extensive thrombosis of the perialveolar capillaries. Autopsies of COVID-19 patients report mononuclear cell infiltrates around thrombosed small vessels and neutrophils within the platelet-rich fibrin thrombi. In addition, resident lung MKs were increased and actively producing platelets [14,39]. Notably, young platelets have increased coagulation potential [14]. Activated platelets bind to leukocytes and promote leukocyte activation and extravasation. They also form aggregates with neutrophils and induce neutrophils to form neutrophil extracellular traps (NETs) [12]. NETS have many functions, including prothrombotic activity. NETs were markedly increased in the blood of COVID-19 patients and were noted in the pulmonary capillaries of COVID-19 acute lung injury [12]. Thus, resident lung MKs may be adding highly thrombogenic platelets to an already hypercoagulable environment [42]. Importantly, previous studies have found that by inducing platelet depletion with TPO silencing, inflammatory immune cells, including monocytes and macrophages, are reduced in the lung. This is in addition to bone marrow mediated platelet and MK lowering effects induced by TPO silencing [35]. Thus, TPO silencing may offer many potential therapeutic advantages in treating thromboinflammation associated with COVID-19.

4. Conclusion

TPO silencing has been shown to safely reduce platelet count and levels of thrombosis in a multitude of inflammatory conditions. Given the extensive role of MK and platelet activity in pathologies associated with thromboinflammation, such as the novel COVID-19, myeloproliferative disorders, malignancy, and autoimmune disorders, TPO silencing may be a potential therapeutic target for these conditions. Further studies are required to better elucidate any adverse outcomes related to TPO silencing in these disease conditions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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