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Platelets in Systemic Sclerosis: the Missing Link Connecting Vasculopathy, Autoimmunity, and Fibrosis?

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Abstract

Purpose of Review Platelets are no longer recognized solely as cell fragments regulating hemostasis. They have pleiotropic functions and they are linked directly or indirectly with the three cornerstones of systemic sclerosis (SSc): vasculopathy, autoimmunity, and fibrosis. In this review, we summarize the current knowledge on the potential role of platelets in the pathogenesis of SSc.

Recent Findings Experimental evidence suggests that vasculopathy, a universal and early finding in SSc, may activate platelets which subsequently release several profibrotic mediators such as serotonin and transforming growth factor β (TGFβ). Platelets may also cross-react with the endothelium leading to the release of molecules, such as thymic stromal lymphopoietin (TSLP), that may trigger fibrosis or sustain vascular damage. Finally, activated platelets express CD40L and provide costimulatory help to B cells, something that may facilitate the breach in immune tolerance.

Summary Preclinical studies point to the direction that platelets are actively involved in SSc pathogenesis. Targeting platelets may be an attractive therapeutic approach in SSc.

Keywords Systemic sclerosis · Scleroderma · Platelets · Serotonin · TGFβ · PDGF · VEGF · Microparticles

Introduction

Platelets are anucleate, cytoplasmic fragments of progenitor bone marrow cells called megakaryocytes [1]. They have a short lifespan of only 7–10 days, and approximately 100 billion platelets are produced from megakaryocytes daily and released in the systemic circulation in order to keep an average count of 1.5–4.5 × 10^5 platelets per microliter of blood [2]. The main role of platelets is to inspect and preserve the integrity of the vessel wall. When the vessel wall is damaged, several soluble factors are released, activating platelets to form a thrombus. Platelet activation can also result from the direct contact of the subendothelial matrix with various glycoprotein receptors of the platelet membrane [3].

In healthy humans, platelet counts are much higher than that needed to prevent bleeding. A number of 0.5 × 10^5 functional platelets per microliter is generally considered a safe threshold, even for major surgeries. However, a healthy organism keeps a fivefold larger number of platelets under normal circumstances; this number can be increased even more in case of stressful conditions [4]. In addition, platelets are equipped with a complex system of granules that contain several molecules not exclusively related to hemostasis. Alpha granules are the main source of hemostatic factors, such as glycoprotein IIb/IIIa and fibronectin, but also contain a number of pro-inflammatory cytokines, such as platelet factor-4 and growth factors, such as platelet-derived growth factor (PDGF) and TGFβ [5]. Dense granules are smaller than α-granules and fewer in number; they are rich in calcium, adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, and histamine. These factors mainly contribute to platelet activation and aggregation. However, serotonin

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besides its well-known vasoconstrictive properties has also been implicated in a wide range of biologic functions unrelated to vascular biology and hemostasis [6–8]. Finally, lysosomes are rich in proteolytic enzymes such as cathepsins, elastases, collagenases, and glycosidases. The functional role of platelet lysosomes is not well understood, but it has been postulated that they contribute to the regulation of thrombus formation and remodeling of the extracellular matrix [9].

The traditional view that platelets are solely cell fragments participating in hemostasis has long been challenged by several facts. Firstly, healthy individuals spend energy to maintain platelet counts way above the necessary threshold to prevent bleeding. Secondly, platelets contain and release molecules unrelated to hemostasis. Nowadays, platelets are thought to have pleiotropic functions and play a role not only in hemostasis but also in immune responses and tissue remodeling following injury. Of note, wherever there is tissue damage there is also vascular damage. This will eventually lead to platelet activation and release of several growth factors which will guide tissue remodeling and repair. Therefore, platelets could be seen as "smart carriers" of growth factors to sites of tissue injury in order to orchestrate the healing process [10–12].

Systemic sclerosis (SSc) carries the highest morbidity and mortality rate among all systemic rheumatic diseases. It is an orphan disease with no approved therapies so far, even though significant progress has been made in the recent past [13–17]. The disease is characterized by the accumulation of collagen into the skin and internal organs leading to fibrosis and eventually organ dysfunction alongside with vascular involvement, manifesting as Raynaud's, pulmonary hypertension, digital ulcers, or scleroderma renal crisis. Raynaud's is usually the earliest event in the pathophysiologic process along with the disruption of the immune tolerance and the presence of autoantibodies. Even though the pathogenesis of SSc is not well understood, it is generally considered that autoimmunity, vasculopathy, and fibrosis are the three cornerstones. Considering the close relationship of platelets with the endothelium and the presence of numerous growth factors and immunomodulatory molecules in their granules, it is not surprising that interest has emerged about the potential role of platelets in the pathogenesis of SSc. Platelets are either directly or indirectly implicated in all three pathophysiologic processes (autoimmunity-vasculopathy-fibrosis) of SSc pathogenesis (Fig. 1). In this review, we summarize the existing evidence related to the contribution of platelets in the pathogenesis of SSc, and we discuss the future research agenda and the potential role of platelets as a therapeutic target in SSc.

Search Strategy

A PubMed search was performed according to published guidance on narrative reviews using the following terms: systemic sclerosis, scleroderma, platelets, fibrosis, Raynaud, interstitial lung disease, and pulmonary arterial hypertension [18]. Original research papers and review articles focusing on the potential role of platelets in pathogenesis and therapy of SSc registered until the end of December 2018 were selected to be included in this review. Priority was given to studies published in the last 5 years. Publications not in English were excluded.

Data Implicating Platelets in SSc Pathogenesis

Platelets as Sources of Profibrotic Mediators

Platelets contain several molecules with profibrotic properties in their granules. These molecules may act as links connecting vasculopathy and fibrosis in SSc; endothelial dysfunction may lead to platelet activation and subsequent release of profibrotic mediators that can stimulate nearby fibroblasts to produce excess amounts of collagen. In this section, we will discuss each one of these molecules.

Serotonin

Serotonin (5-HT) is a molecule with pleiotropic functions produced by either the brain (brain-derived serotonin (BDS)) or the duodenum (gut-derived serotonin (GDS)). BDS plays a key role as a neurotransmitter in the central nervous system. GDS is mainly stored in platelets and is a powerful vasoconstrictor; it is released by activated platelets following vascular damage and facilitates hemostasis by causing vasoconstriction. However, strong experimental evidence implicates serotonin in many other biologic functions including tissue remodeling/fibrosis [19, 20].

Dees et al. first proposed that serotonin released from platelets can be the crucial link between vasculopathy and fibrosis in the pathogenesis of SSc. The investigators extracted skin fibroblasts from SSc patients and healthy subjects; cultured fibroblasts were then treated with serotonin. They found that serotonin enhances the production of extracellular matrix in both scleroderma and normal fibroblasts. They also showed that this effect was mediated by the 5-HT_{2B} receptor through in a TGFβ-dependent manner. Further experiments by inhibiting 5-HT_{2B} signaling using the inhibitor terguride, in two animal models of SSc, showed decreased dermal thickening in both models. In addition, blocking platelets with the P2Y12 receptor inhibitor clopidogrel, reduced the serotonin content in the fibrotic skin in both models. Consistently, collagen content and myofibroblast counts were also significantly reduced [6]. The stimulatory effect of serotonin on fibroblasts shown in the study by Dees et al. has been recently verified by another research group. Chaturvedi et al. studied the effect of serotonin on cultured fibroblasts extracted from a patient with SSc and found that serotonin upregulated the expression of profibrotic genes and the production of collagen. In an effort to further explore how serotonin mediates these profibrotic effects,

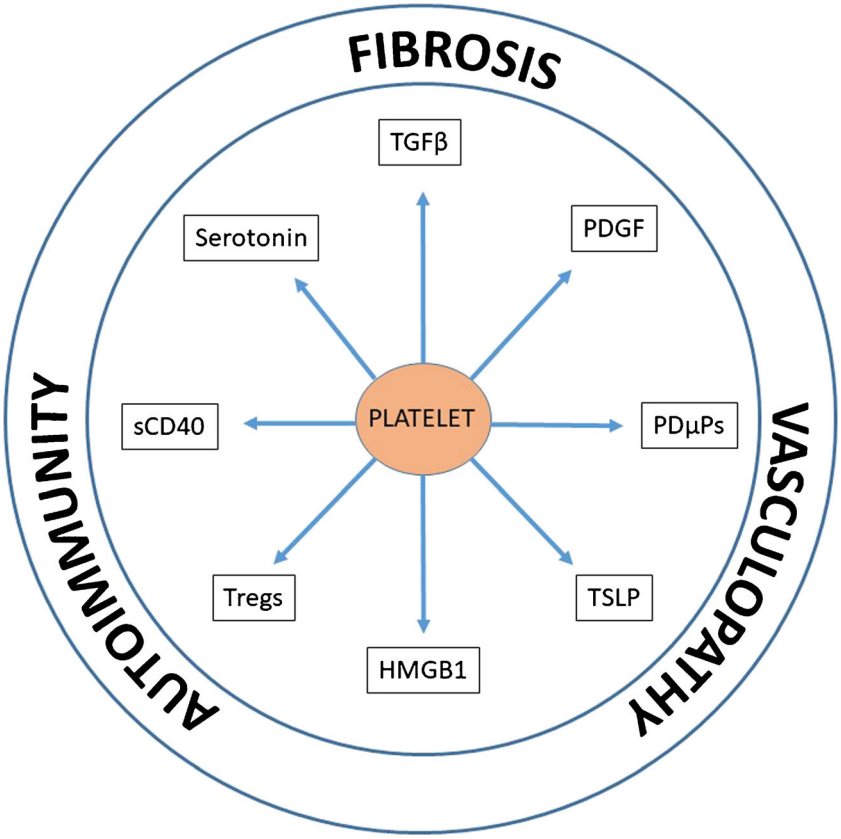

Fig. 1 Platelets may participate in all three key pathophysiologic processes of SSc (vasculopathy-autoimmunity-fibrosis) by several ways: (i) by secreting profibrotic mediators such as serotonin, TGFβ, and PDGF, (ii) by cross-reacting with the endothelium and releasing molecules such as thymic stromal lymphopoietin (TSLP), HMGB1 or microparticles that may trigger fibrosis, or sustain vascular damage, and (iii) by providing costimulatory help to B cells through a CD40L-dependent manner or interacting with Tregs

the authors performed additional blocking experiments by using two different serotonin receptor inhibitors (terguride and SB204741, inhibitors of 5-HT_2, and 5-HT_{2B} respectively) and found that both inhibitors were able to reverse the effects of serotonin on cultured fibroblasts and to reduce the expression of profibrotic genes. The investigators also showed that serotonin receptor inhibition attenuates the expression of TGFβ1-related genes and collagen production [21]. These data clearly indicate that serotonin has a profibrotic role by stimulating fibroblasts and propose an intriguing pathogenetic model. Vasculopathy and endothelial dysfunction are very early events in the pathophysiologic process of SSc. This is underscored by the fact that Raynaud's, a typical vascular manifestation, is almost always present for years prior to any other symptom or sign in patients with SSc. Vasculopathy may lead to platelet activation and local serotonin release; serotonin may act on nearby fibroblasts and trigger fibrosis. This pathogenetic model can explain why skin fibrosis in SSc always starts from the fingers in the form of sclerodactyly; the fingers are sites first affected by vasculopathy.

TGFβ

TGFβ, the most powerful profibrotic mediator known in humans, was initially discovered in platelets [22]. It is a key player in the wound healing process and normally, it is upregulated following tissue damage and plays a pivotal role in the production of extracellular matrix. TGFβ has been long implicated in fibrosis and it is generally considered as a central mediator of SSc pathogenesis [23]. Two distinct forms of TGFβ are recognized: a latent and an active form. Platelets are a significant source of TGFβ but also contain activators of latent TGFβ to its active form [24, 25]. Conversely, TGFβ also enhances platelet aggregation [26]. Therefore, it is reasonable to hypothesize that platelets are activated following contact with the damaged endothelium in patients with SSc and subsequently, release latent TGFβ. Platelets have the tools to convert latent TGFβ to its active form which can stimulate nearby fibroblasts and trigger the fibrotic process. Moreover, TGFβ may enhance platelet activation leading to further TGFβ release and vascular damage thus creating a vicious circle with potential pathogenetic implications in SSc.

Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF), discovered in the 1970s as a serum factor that stimulates proliferation of fibroblasts and smooth muscle cells, originates mostly from platelets. It is considered that PDGF is working in concert with TGFβ in the development of organ fibrosis [27]. The key role of PDGF in fibrosis is underscored by the fact that mice genetically engineered to overexpress PDGF develop a fibrotic phenotype resembling SSc [28]. In humans, overexpression of

PDGF has been shown in both skin and lungs of patients with SSc [29]. Platelets are considered as the most significant source of PDGF.

There is strong experimental evidence that PDGF and TGFβ are key molecules in SSc. Even though both molecules are stored in large amounts in platelets, the contribution of platelet-derived PDGF and TGFβ in the pathogenesis of SSc is not known because direct experimental evidence is lacking.

Platelets and Vasculopathy

The Platelet-Endothelium Cross Talk in the Pathogenesis of SSc

The interplay between platelets and the endothelium leads to the release of several molecules that may trigger fibrosis or sustain vascular damage, events critical in SSc pathogenesis.

Thymic Stromal Lymphopoietin

Thymic stromal lymphopoietin (TSLP) is an interleukin-7 cytokine family member which promotes Th2 differentiation and has been implicated in the pathogenesis of idiopathic pulmonary fibrosis [30, 31]. Truchetet et al. recently proposed that TSLP may act as a link between platelets and skin fibrosis in SSc. They explored the hypothesis that platelets may contribute to skin fibrosis via activation of human dermal microvascular endothelial cells (HDMECs) which subsequently produce TSLP. They performed in vitro experiments and showed that ADP-activated platelets from healthy donors induce TSLP production by HDMECs in an interleukin-1b and serotonin-dependent manner. Further, in vitro experiments showed that TSLP has profibrotic properties by promoting collagen production from fibroblasts. In the clinical setting, the investigators found that TSLP is increased in the sera and the skin of SSc patients, especially when digital ulcers are present. Considering that platelets are in a continuous active state in SSc due to underlying vascular disease, the aforementioned mechanism could represent another pathogenetic link between vasculopathy and fibrosis [32••]. Vascular damage induces platelet activation and conversely, platelets through interleukin-1b and serotonin release, induce overexpression of TSLP in endothelial cells, which contributes to overproduction of collagen from nearby fibroblasts.

High-Mobility Group Box 1

High-mobility group box 1 (HMGB1) is a prototypic damage-associated molecular pattern (DAMP) that has been implicated in the pathogenesis of SSc [33]. Platelets contain this molecule in their microparticles. Platelet-derived microparticles (PDμPs) are membrane fragments that arise when platelets undergo activation and apoptosis and represent a mean of platelet communication with other cells. Maugeri et al. proposed that hypoxia and reactive oxygen species (ROS) elicit a platelet-neutrophil cross talk that subsequently leads to vessel damage through the release of microparticles rich in HMGB1 by activated platelets. PDμPs from SSc patients were shown to express higher amounts of HMGB1 compared to healthy controls. Neutrophils incubated with activated platelets or PDμPs from SSc patients showed increased activity compared to neutrophils incubated with resting platelets or PDμPs from healthy subjects. This effect was attenuated by HMGB1 antagonists or reducing agents, suggesting that HMGB1 in an oxidative milieu is responsible for neutrophil activation. These results point to the direction that platelets and neutrophils in SSc may participate in a vicious cycle, where activated platelets stimulate neutrophils to generate ROS which in turn oxidase platelet HMGB1 and further activate neutrophils, resulting in vessel and tissue damage [34].

A recent study by the same team showed that PDμPs from patients with SSc activate autophagy in neutrophils and the generation of neutrophil extracellular traps (NETs). The investigators showed that PDμPs from SSc patients induce autophagy of neutrophils in a HMGB1-depended manner, in contrast to PDμPs from healthy donors or lupus patients. Addition of a HMGB1 antagonist attenuated this effect. They also injected collagen-activated human PDμPs to mice to examine the in vivo effect of PDμPs on neutrophils and they found increased numbers of circulating autophagic neutrophils. Finally, they showed that mice injected with PDμPs derived from SSc patients displayed increased endothelial activation, pulmonary damage, perivascular inflammatory infiltration, and increased pulmonary collagen accumulation compared to mice injected with PDμPs from control subjects [35••]. These data suggest that PDμPs may participate in SSc pathogenesis by inducing and sustaining endothelial damage.

Vascular Endothelial Growth Factor and VEGF165b

Hirigoyen et al. studied the effect of platelets obtained from either patient with SSc or healthy subjects on HDMECs. Commercially available HDMECs were used in an assay that cells undergo tubule formation exhibiting capillary-like structures. Platelet-rich plasma was obtained from SSc patients and healthy donors, and following centrifugation, the supernatant was isolated and several angiogenic and inflammatory mediators were measured; these included TGFβ, CD40L (a marker of platelet activation), tumor necrosis factor (TNF-α), vascular endothelial growth factor (VEGF), its antiangiogenic isoform VEGF165b, and connective tissue growth factor (CTGF). The authors found significant increases in basal TGFβ and CD40L in the supernatants of platelets from patients with SSc compared to supernatants of platelets from healthy control subjects. The supernatants from platelets

obtained from patients with SSc significantly inhibited tubule formation in HDMEC cultures compared to platelet supernatants from healthy control donors. Finally, the antiangiogenic isoform VEGF165b was increased in SSc platelet supernatants compared to healthy controls. These data suggest that platelets in SSc are in an active state and may contribute through the secretion of VEGF165b to angiogenesis inhibition and microangiopathy of SSc and through the secretion of TGFβ to fibrosis [36].

Platelets and Pulmonary Arterial Hypertension

Platelets seem to be active players in the development of vasculopathy in SSc. Platelets and pulmonary arterial hypertension (PAH) is a vascular manifestation appearing later on the disease course with a major impact on mortality and quality of life. There are several experimental data implicating platelets in the pathogenesis of PAH. Firstly, patients with PAH have abnormalities in the number of circulating platelets and the mean platelet volume could have a prognostic value or reflect disease severity [37, 38]. Moreover, thrombotic lesions are common histologic findings in PAH, even though the pathogenetic significance of this is not yet clear [39•].

Secondly, several platelet-derived molecules are impaired in PAH. For example, platelets from patients with PAH have lower levels of soluble TWEAK (tumor necrosis factor-like weak inducer of apoptosis). Lower levels of soluble TWEAK also correlate positively with PAH progression [40]. TWEAK is a member of the TNF superfamily of cytokines which normally regulates various physiological processes and seems to play an important role in tissue repair following acute injury [41]. Another example is platelet microparticles which are elevated in patients with PAH or Raynaud's and could play a pathogenetic role due to their pro-inflammatory properties [42]. Furthermore, platelet activation and release of serotonin could contribute to the vascular remodeling in PAH and SSc as an adjunct stimulus important for the proliferation processes which lead to vessel narrowing [43•]. Disturbances in platelet markers of activation are common among patients with PAH or primary Raynaud phenomenon, possibly reflecting their exhaustion from continuous stimulation [44, 45].

Thirdly, Toll-like receptor 4 (TLR-4) is implicated in the pathogenesis of PAH and recently, Bauer et al. showed that deletion of this particular receptor from platelets in two mouse models of PAH protected against the development of pulmonary hypertension. In sharp contrast, TLR-4 deletion from any myeloid lineage cell was not protective [46].

Finally, platelets can physically interact and bind to smooth muscle cells, through the integrin receptor $\alpha_{IIb}\beta_3$, and affect their proliferation and migration in vitro, factors important for vascular remodeling [47]. The above data indicate that platelets may participate in the pathophysiologic process of PAH and suggest that they could be targeted therapeutically. Targeting platelets and platelet-derived serotonin in PAH has given some positive results in animals but studies in humans have not yielded conclusive results indicating the need for further research [48]. Currently, antiplatelet therapy is not routinely recommended in SSc-related PAH [49].

Platelets as Immunomodulators

It is now well recognized that platelets are able to modulate innate and adaptive immune responses. There is significant evidence indicating that platelets regulate inflammatory responses during acute coronary syndrome, infections, and cancer [50, 51]. Moreover, platelet abnormalities and aberrant activation are frequently found in several autoimmune diseases [12]. Whether platelets directly or indirectly contribute to pathogenesis of rheumatic diseases by enhancing immune activation or promoting tissue and vascular damage is not clear, but evidence is pointing to an active pathogenic role. Recent studies have given new insights into the immune properties of platelets. There are two main mechanisms through which platelets may modulate immune responses. Firstly, platelets may interact with T regulatory cells (Tregs) and secondly, platelets can affect monocytes or B cells via the costimulatory axis CD40/CD40L.

Tregs have a pivotal role in balancing inflammatory responses in autoimmune diseases via suppression of pathogenic immune pathways. In a murine model of tissue damage, Bergmann et al. showed that Treg responses following injury were affected by platelet depletion. Following burn injury, platelet depletion reduced activation of Tregs. Conversely, depletion of Tregs resulted in a diminished hemostatic capacity of platelets, indicating a cross talk between platelets and Tregs [52•]. Platelets may also affect Tregs recruitment through CD40-dependent mechanisms [53]. Moreover, direct binding of platelet microparticles to Tregs has been shown to affect their proliferation and differentiation in vitro [54•]. The potential cross talk between platelets and T cells may have a pathogenetic role in the early stages of SSc, where platelet aggregates and infiltrates of T cells and monocytes are the main histologic findings in skin biopsies [55, 56].

Platelets are able to release soluble CD40L and this is a potential mechanism to modulate responses from monocytes or B cells [57]. Soluble CD40L is released from alpha granules upon activation. It has been shown in vitro that platelets interact with B cells through CD40L and enhance the production of IgG class antibodies [58]. In vivo studies have confirmed the ability of platelets to provide costimulatory help to B cells [59]. Similarly, platelet microparticles generated following platelet activation are also carriers of CD40L and can mediate costimulatory help to B cells [60]. Another report provides evidence that platelets from immune thrombocytopenic purpura patients can drive the activation of autoreactive B cells, via increased expression of CD40L [61]. This data support the

hypothesis that activated platelets in the periphery can facilitate the activation of B cells, via generation of soluble CD40L and microparticles. In the setting of SSc pathogenesis, these stimuli from activated platelets may facilitate the disruption of immune tolerance and the production of autoantibodies.

Targeting Platelets in SSc

The need for effective therapies in SSc remains urgent, albeit the recent advances. Platelets appear promising targets for novel therapies due to their pleiotropic functions and the close cross talk with cells of the immune system. However, a preliminary controlled trial of the combination of aspirin and dipyridamole 30 years ago yielded discouraging results and weakened the interest in further exploring antiplatelet therapy in SSc [62]. The same combination was not efficacious in a more recent uncontrolled trial, aiming to reduce the burden of Raynaud's attacks in patients with SSc [63]. Hydroxychloroquine has recently shown some efficacy in SSc regarding articular involvement [64]. Of note, hydroxychloroquine is well known to exhibit antiplatelet effects [65, 66]. Iloprost and riociguat also have inhibitory effects on platelets and this may contribute to their action in SSc [67, 68]. Terguride, a selective serotonin receptor inhibitor, has positive preliminary results in patients with SSc. In a proof of concept study, 18 patients were recruited; 12 into the terguride group and 6 into the placebo group. Skin biopsies showed significant reduction of markers of fibrosis and MRSS improved in the terguride group after 3 months of treatment. Major safety issues were not observed [69••]. Based on these preliminary positive results, terguride is currently tested in a phase III multicenter trial in diffuse SSc (TERGSIS study, EudraCT Number: 2015-002586-39).

The effect of clopidogrel in the course of SSc was explored in our department in a small uncontrolled trial of 13 patients. We did not detect significant reduction in circulating serotonin and found no evidence of clinical benefit. In fact, three of the 13 patients developed digital ulcers while on treatment. Additionally, markers of endothelial dysfunction deteriorated. Interestingly, 2 of the 3 patients who developed digital ulcers while on treatment had no history of ulcers in the past. The potential association of digital ulcers with platelet inhibition led us to an early termination of the study [70]. On the other hand, bearing in mind that platelets are highly activated in SSc, trials of newer antiplatelet drugs or combination regimens may lead to different results. Of interest, ifetroban, an oral thromboxane-A_2 receptor antagonist is currently evaluated in a phase II clinical trial in diffuse SSc- and SSc-associated PAH (NCT02682511).

Conclusion

The concept that platelets may act as effector cells in autoimmunity, vascular damage, and fibrosis in SSc is not a new one. Platelets are able to interact with lymphocytes, fibroblasts, and endothelial cells, all key cells in SSc pathogenesis. Most studies agree that platelets are in a continuous active state in SSc and preclinical data from animal models suggest a potential pathogenetic role. However, antiplatelet regimens tried in small series of patients with SSc have failed to show any clinical benefit thus far. A possible explanation for this discrepancy could be the short duration of these trials, bearing in mind that platelets are just one component in the vicious cycle of fibrosis. One could also assume that the generalized vasculopathy of SSc demands a more potent inhibition of platelets, in order for clinical benefit to be achieved.

Most preclinical and clinical data suggest that specific molecules stored and released from platelets participate in the vicious cycle of autoimmunity-vasculopathy-fibrosis. Direct inhibition of these molecules may be an alternative to the use of classic antiplatelet drugs. Serotonin has the most robust data as a potential target among the platelet-derived molecules.

Terguride seems a promising agent that is currently been evaluated in a phase III clinical trial. A thromboxane-A_2 receptor inhibitor is also under evaluation in a smaller phase II trial. Other agents, such as TSLP, HMGB1, CD40L, and VEGF165b could represent novel targets in the future. Inhibitors of these molecules are currently tested in various diseases. The results of all these trials are expected with interest. They could provide new insights into the potential contribution of platelets in SSc along with the establishment of new therapeutic options.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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