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Thrombospondins: Old Players, New Games

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Abstract

Purpose of review—Thrombospondins (TSPs) are secreted extracellular matrix (ECM) proteins from TSP family, which consists of five homologous members. They share a complex domain structure and have numerous binding partners in ECM and multiple cell surface receptors. Information that has emerged over the last decade identifies TSPs as important mediators of cellular homeostasis, assigning new important roles in cardiovascular pathology to these proteins.

Recent findings—Recent studies of the functions of TSP in the cardiovascular system, diabetes and aging, which placed several TSPs in a position of critical regulators, demonstrated the involvement of these proteins in practically every aspect of cardiovascular pathophysiology related to atherosclerosis: inflammation, immunity, leukocyte recruitment and function, function of vascular cells, angiogenesis, and responses to hypoxia, ischemia and hyperglycemia. TSPs are also critically important in the development and ultimate outcome of the complications associated with atherosclerosis – myocardial infarction and heart hypertrophy and failure. Their expression and significance increase with age and with the progression of diabetes, two major contributors to the development of atherosclerosis and its complications.

Summary—This overview of recent literature examines the latest information on the newfound functions of TSPs that emphasize the importance of ECM in cardiovascular homeostasis and pathology. The functions of TSPs in myocardium, vasculature, vascular complications of diabetes, aging and immunity are discussed.

Keywords

thrombospondin; extracellular matrix; cardiovascular system

Introduction

The history of our understanding of the functions of thrombospondins (TSP) is a great example of the typical evolution of our knowledge of any molecular entity: as we explore their properties and roles in various models and systems, we learn about the incredible complexity of their interactions and functions and their impressive influence in a number of pathophysiological processes.

TSPs are secreted extracellular matrix (ECM) proteins whose functions in many tissues, including cardiovascular system, were obscure until recently. Transgenic mice with knockouts of each TSP have been generated, and none of the knockouts resulted in an overt phenotype that would lead to death or disease in the unchallenged young animal. However, the recent reports about individual proteins of this family revealed not only the complexity

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of their regulation, functions and interactions, but also has once again demonstrated the nature's amazing "no waste" policy: we learned that their physiological and pathological "work" starts in the ER as they are being processed and secreted [1**] and continues on the cell surface and in the extracellular matrix (ECM), where their multi-functionality unfolds.

This article provides an overview of recent publications that suggest novel functions and mechanisms of TSPs' regulation and signaling in cardiovascular system. Recent information regarding functions of TSPs has forever changed our view of these ECM proteins as unnecessary components of ECM and placed this protein family among the most active and potent regulators of important homeostasis and pathologies.

The family history: TSP protein family

The thrombospondin family includes five members – TSP-1, TSP-2, TSP-3, TSP-4, and TSP-5 or COMP (Cartilage Oligomeric Matrix Protein). They have been assigned to the same family based on the similarities in their modular protein domain structure. However, all five are the products of different genes from different chromosomes, and the regulatory DNA and mRNA regions of five genes differ. As a result, their spatial and temporal expression profiles are distinct, suggesting differential non-overlapping functions.

Although the similarity between TSPs has been repeatedly stressed, there have been very few attempts to compare the proteins side by side in the same experimental model, with the exceptions of TSP-1 and TSP-2 [e.g., 2-4]. One could expect that the similarity in the protein organization and structure would compel investigators to examine the expression of all five proteins in each of the individual TSP knockout mice and to study the feed-back regulation and possibility of compensation or to look for the common properties between five TSPs. Amazingly, this was not the case. Instead, most TSP studies focused on an individual TSP protein.

TSP family is divided in two subfamilies – A (TSP-1 and TSP-2) and B (TSP-3, TSP-4, and TSP-5) (Fig.1) [5]. This classification is based on TSP domain structure and their mode of multimerization (TSP-1 and TSP-2 are trimers, while the rest of TSPs are pentamers). The analysis of evolutionary patterns revealed that the subgroup B TSPs appeared earlier and that the subgroup A is the product of most recent evolution [6].

TSP-1 and TSP-2 are produced by a number of cells in response to injury and during remodeling (reviewed in [2, 7-9]. TSP-1 was discovered first, and a large body of literature documented its importance in regulation of angiogenesis and thrombosis (e.g., [10-16]).

TSP-5 and TSP-3 are abundant in the vascular wall [17], but their functions and significance of their expression in vasculature are unclear. TSP-3 is the least studied of all five family members, and its functions in the site of high expression [vascular wall, tendon] have not been systematically examined.

TSP-4 was poorly studied until about a decade ago, but has received more attention in the recent years.

The most impressive recent progress in our understanding of TSP functions and physiological significance has been achieved in studying the cardiovascular system. The interest in TSP functions in myocardium and vasculature was rekindled by a publication of the results of a genetic association study [18] that reported three coronary-artery-disease (CAD)- and myocardial-infarction (MI)- associated single nucleotide polymorphisms (SNPs) in TSP-1, TSP-2 and TSP-4 genes. Several follow-up reports confirmed these associations [18-24], while others failed to reproduce these results [25], presumably, either

due to a difference in the selection of participants or the design of the study (reviewed in [5, 26]). The cardiovascular functions of all three TSPs were studied in animal KO models, and important conclusions were reached regarding their importance in the myocardium and in the vascular wall.

Integrity of heart: TSPs in heart

All five TSPs are expressed in healthy myocardium, and the increased expression of each individual TSP has been detected in remodeling hearts: the published reports documented increased levels of TSP-1, TSP-2 and TSP-4, and our unpublished study revealed higher expression of TSP-3 mRNA and TSP-5 mRNA and protein in pressure overloaded mouse hearts [9, 27-36].

TSP-1, TSP-2 and TSP-4 regulate fibrosis [28*, 37-39*]. However, TSP-1 and TSP-2 upregulate, but TSP-4 downregulates the fibrosis in myocardium during remodeling, as became clear from observations in human heart allografts [31, 39*] and from animal models of heart remodeling and repair [28*, 37, 38*]. In human heart allografts, increased expression of both TSP-1 and TSP-2 was an indicator of the fibrotic response and the rejection [31, 39*]. When TSP-1 and TSP-2 KO mice were tested in myocardium remodeling models, e.g., diabetic cardiomyopathy [40], doxorubicin-induced cardiomyopathy [35], or MI [32, 41, 42], their role in promoting pro-fibrotic changes in remodeling myocardium was confirmed. The pro-fibrotic activity of TSP-1 and TSP-2 results from a combination of their properties: inhibition of MMPs [39*, 42, 43], activation of TGF-beta by TSP-1 [39*, 44*], the anti-angiogenic effects [9, 31, 38*, 42] all seem to contribute to fibrosis, but TSP-4 directly effects cellular functions in myocardium [1**]. TSP-1 also signals through calreticulin/LRP1 complex to activate cell-survival signals in fibroblasts and to increase their migration [45], and this mechanism may also contribute to pro-fibrotic activities of TSP-1 during repair and remodeling processes.

TSP-2 was identified as a marker of an early-stage progression from compensated heart hypertrophy to heart failure [43]. The response of *Thbs2*^{-/-} mice to angiotensin II suggests that TSP-2 is a crucial regulator of the integrity of the cardiac matrix and is required for the myocardium to cope with overloading: 70% of mice had a fatal cardiac rupture, and the rest of them developed cardiac failure [43].

All three TSPs of group B (TSP-3, TSP-4, and TSP-5) are expressed in the remodeling heart [1, 27, 28*, 30, 37, 46, 47]. However, TSP-4 deficiency has an effect that is opposite to the effects of TSP-1 or TSP-2 deficiency in remodeling hearts: it increases fibrosis [28*, 37]. In addition to the anti-fibrotic effect of TSP-4, it also supports the adaptation of myocardium under stress by augmenting intracellular myocyte Ca and improving contractility [37]. A new unexpected function of intracellular TSP-4 in endoplasmic reticulum (ER) stress response, which may be present in other TSP-4-expressing cells as well, was discovered in cardiomyocytes: TSP-4 interaction with the ER luminal domain of activating transcription factor 6α (Atf6α) produced a unique profile of adaptive ER stress response factors and protected myocardium from pressure overload [1**]. Thus, the increased levels of TSP-4 in failing human hearts and experimental remodeling animal heart models must be considered as a protective response in damaged and remodeling myocardium. TSP-4 seems to directly inhibit the production of collagen [28*] through unknown signaling in fibroblasts and other cell types.

The reasons for the dramatic difference in the effects of TSP-1/TSP-2 and TSP-4 on fibrosis in myocardium are unclear: the effects have not been attributed to specific domains of TSPs or TSP receptors. TSP-4 lacks the domains analogous to TSP-1 and TSP-2 domains regulating angiogenesis, MMP activity and TGF-beta activation (Fig.1), and the differential

effects may be due to this structural difference and, as a result, a differential interactions with cell surface receptors and the binding partners in ER or ECM. New clues regarding specific contributions of TSPs in the remodeling process and regarding the specific functions and significance of each TSP in myocardium could be obtained from monitoring and manipulating individual TSP expression at different stages of the remodeling and response to injury, which has yet to be done.

All four thrombospondins were detected in aortic valves, and increased levels of TSP-2 were associated with fibrosclerosis and stenosis [48**]. The highest expression of TSP-4 in the normal heart was detected in valves, suggesting that this protein also plays an important structural or functional role in these areas [28*].

Blood is thicker than water: blood vessels and TSPs

Although TSPs appeared in early metazoans [5, 49], their development into TSPs similar to the ones we now see in humans (into group B and later into group A TSPs) coincides with the development of circulatory system. Indeed, all TSPs are present in blood vessels [17], and TSP-1, TSP-2 and TSP-4 have been proved important in regulating the functions and the structure of the vascular wall and its interactions with the blood cells.

TSP-1 and TSP-4 knockout mice were examined in a mouse atherosclerosis model (*ApoE*^{-/-}) [17, 50]. In both knockouts, the development of atherosclerotic lesions was slower in the early stages and in less inflammatory conditions. However, the lesions were the same size as the lesions in control animals at later stages with the well- developed inflammatory plaques, as was judged by the lipid staining. Neither of two proteins changed the accumulation of lipids in the lesion over time, but the deficiency in either TSP-1 or TSP-4 resulted in profound changes of the lesion composition, affecting either the function (TSP-1) or the recruitment (TSP-4) of macrophages in the lesion. TSP-1 deficiency caused a more inflammatory lesion due to the inhibition of phagocytosis in macrophages, suggesting that TSP-1 plays a protective role in late inflammatory lesions by stimulating the phagocytosis [50]. In TSP-4 knockout mice, the lesions showed less inflammation and fewer macrophages, as well as a reduction in the production of inflammatory cytokine [17], suggesting that TSP-4 promotes local vascular inflammation (Fig.2). In *in vitro* settings, TSP-4 promoted adhesion and migration of both microphages and neutrophils [17, 51], and integrin $\alpha_v\beta_3$ was identified as one of the receptors mediating these effects. Interestingly, the mutant TSP-4 carrying the SNP associated with CAD and MI [18] was more active in promoting leukocyte adhesion and migration, as well as intracellular pro-inflammatory signaling in vascular and blood cells, apparently due to its ability to bind more Ca²⁺ resulting in altered conformation allowing better interaction with the cell surface receptors [52]. These observations offer insights into the mechanisms of the genetic association between TSP-4 and CAD/MI.

In the vascular wall, regulation of fibrosis did not seem to be involved in the effects on atherogenesis: TSP-1 deficiency promoted the fibrous cap formation, but this did not determine the fate of the lesion, and the effect of TSP-1 on macrophage function was more important [50]. TSP-4 deficiency did not result in altered matrix production in the lesion [17].

The interaction of TSP-1 and its receptor CD47 has recently received a lot of attention due to the importance of this pathway in regulation of vascular resistance, leukocyte functions, and intracellular signaling in EC and VSMC. Based on the structural studies of the TSP-1 fragment bearing the CD47 binding site, it was suggested that this site is deeply buried in a hydrophobic pocket and should not be available to interact with the cell surface receptor [53]. However, it became obvious that such interaction does occur in cellular systems [54,

55] and that this interaction is very significant in regulation of physiological and pathological cardiovascular responses [56-65]. The interaction of TSP-1 and CD47 inhibits the canonical nitric oxide pathway, regulates blood pressure and maintains global hemodynamics under stress [60], and contributes to regulation of hemostasis, immunity and mitochondrial function (reviewed in [66**]). The binding site for CD47 is located in the signature domain homologous in all TSPs and it is possible that similar functions may be identified in other TSPs. However, such studies have not been done or have not been reported to date.

The anti-angiogenic functions of TSP-1 and TSP-2 have been known for many years and have been studied in various angiogenesis models and angiogenesis-related diseases (reviewed in [12, 13, 42]). However, it is impossible to talk about TSPs in vasculature without mentioning this important function of two potent anti-angiogenic proteins. In addition to a large body of new reports confirming the association of TSP-1 and TSP-2 levels with angiogenesis and various cancers, the new understanding of the power of TSP-1 to prevent neovascularization has emerged. It became clear that TSP-1 expression in a tissue could determine the fate of angiogenesis even without modulation of pro-angiogenic stimuli [67, 68] and that modulation of TSP-1 production can be a natural mechanism accounting for the tissue-specific fate of angiogenesis [69*].

The anti-angiogenic effects of TSP-1 have been initially attributed to its N-terminal half [70-72]. However, its interaction with CD47 has been recently shown to contribute to anti-angiogenic properties of TSP1 by disrupting the signaling from VEGFR2, for which the direct interaction of VEGFR2 and CD47 is required [73].

3. Little sugar in my blood: diabetes and TSPs

TSP-1 production is highly responsive to the level of blood glucose, and the regulation of TSP-1 by hyperglycemia has been reported in multiple tissues [40, 74-81]. It is not surprising that TSP-1 has been associated with several diabetic complications [40, 69*, 74, 75, 78, 82]: altered angiogenesis, vascular inflammation and altered fibrosis are the hallmark of diabetic complications and cause most of the morbidity and mortality in diabetic patients. The pro-fibrotic properties of TSP-1 mediated by activation of TGF β contribute to the diabetic changes in kidney and in myocardium [40, 75]. We have recently reported a novel cell- and tissue-specific mechanism that regulates TSP-1 production in response to hyperglycemia and may account for the tissue-specific associations of cancers and neovascularization in organs with diabetes [69*]. This pathway is activated in response to hyperglycemia and is mediated by microRNA miR-467, which binds a regulatory element in the untranslated region of TSP-1 mRNA. The binding results in translational silencing and lack of production of TSP-1 and increased neovascularization in the tissues where this mechanism (upregulation of miR-467 by hyperglycemia) is present. This novel mechanism provides a good example of the importance of TSP-1 in maintaining the anti-angiogenic pressure in the tissues and offers an attractive therapeutic target to correct diabetic neovascularization in a tissue- and organ-specific manner.

However, in addition to its role in diabetic vascular complications, TSP-1 mediates development of insulin resistance and glucose intolerance by promoting inflammation and macrophage recruitment in adipose tissue [83].

The recent reports on TSP-1 define this protein as both the mediator of the effects of hyperglycemia and the causative stimulus for development of hyperglycemia. This information places TSP-1 in a position of a major regulator of both the metabolic changes and the responses of vasculature to metabolic perturbations.

4. Age is just a number: aging and TSPs

A number of reports and unpublished observations suggest that the importance of TSPs increases with age. Not only has the higher expression of TSP proteins and their receptors been detected in tissues [28*, 84**, 85], but their presence also has appeared to become more important [28*, 84**-86*]. In the knockout mice that did not have overt differences from their WT littermates at a young age, structural and functional changes develop with aging.

The levels of TSPs and their receptor CD47 increase with age [84*]. It is unclear what causes this increase and whether there are benefits of increased CD47 and TSP-1 levels. Blocking the signaling from TSP-1-CD47 promoted normal function of mitochondria in skeletal muscles and resulted in more efficient metabolism and in the enhanced physical performance, suggesting that (at least in skeletal muscles) the age-related upregulation of TSP-1 and CD47 is not beneficial [87]. Increased levels of TSP-1 and downregulation of nitric oxide pathway may be a contributing factor of multiple diseases of aging, which are characterized by the deficient NO signaling [66**]. TSP-1 is an activator of TGF β , and both TSP-1 and TGF β levels were increased in aging mice along with increased fibrosis and [44*].

TSP-2 is required for cardiomyocyte function and maintenance of a healthy matrix in aged hearts [85]. TSP-2 deficiency resulted in a severe dilated cardiomyopathy with impaired systolic function resulting in higher death rate in aging *Thbs2*^{-/-} mice. Ultrastructural analysis suggested that progressive myocyte death with the reparative fibrosis and the increased inflammatory response were responsible for this phenotype. Abnormal collagen cross-linking and alteration of expression of enzymes controlling ECM, as well as impaired signaling in cardiomyocytes accompanied the disorder, suggesting that TSP-2 in heart protects against age-related cardiomyopathy.

When the association between the levels of TSP-1 and TSP-2 in blood and cardiovascular mortality was studied in elderly men, TSP-1 levels were not associated with mortality, while TSP-2 in blood strongly correlated with mortality after adjusting for other cardiac risk factors [88**].

TSP-1 expression was more sensitive to inflammatory stimulus (LPS) in white adipose tissue of aged animals [86*]. Knowing that TSP-1 in adipose tissue promotes inflammation and insulin resistance [83], this observation can be considered as an indication that TSP-1 may have causative effects in age-related inflammatory and metabolic disorders, including atherosclerosis.

Unchallenged aging *Thbs4*^{-/-} mice develop fibrotic changes in myocardium that are similar to the ones seen in myocardium remodeling in response to pressure overload in young animals [28*]. This observation suggests the beneficial and protective effect of TSP-4 in the aging heart.

5. TSPs and immune system

The immune system contributes to every pathological process mentioned above, and therefore the advancement of understanding the role of TSP in regulation of immune system must be briefly discussed.

TSP-1 negatively regulates T-cell activation [89*, 90]. This effect is mediated by CD47: TSP-1 binding to CD47 prevents the hydrogen sulfide signaling that leads to activation of T-cells.

TSP-2 was protective in viral myocarditis model by promoting the activation of anti-inflammatory T-regulatory cells and inhibited cardiac inflammation and injury [91**]. The effects of TSP-2 on T-cells is also mediated by interaction with CD47 (all thrombospondins share CD47-binding site in their highly homologous signature C-terminal domain) [92].

In vitro, TSPs have been shown to regulate a number of T-cell functions, including adhesion [93], migration [94], differentiation [95] and apoptosis [96].

Both TSP-1 and TSP-4 increase the recruitment of leukocytes in inflammation models [17, 51, 97**], and both proteins upregulate the local inflammation and cause the release of chemokines [17, 97**].

The findings of the effects of TSP-1 on phagocytosis are controversial: while Moura et al reported that TSP-1 promotes phagocytosis by macrophages in atherosclerotic lesions [50], in a candidiasis model TSP-1 inhibited the phagocytic capacity of inflammatory leukocytes, resulting in increased fungal burden [97**]. TSP-1 enhances the survival and reduces autophagy in irradiated T-cells, and this effect is also mediated by CD47 [98**].

Conclusion

Despite the limited scope of this review and the lack of a detailed discussion of the novel functions of TSPs outside the cardiovascular system, the overview of recent reports, although limited, clearly identifies TSPs as important regulators of cellular functions and important pathophysiological processes (summarized in Table 1). While a decade or two ago TSPs may have appeared to be redundant ECM proteins with an unclear significance, recent findings about the interactions and functions of this protein family mark TSPs as critical regulators of cardiovascular functions. The TSP story represents an emerging shift of interest from studying only the intracellular pathways to examining the complex interactions of ECM and cells and to understanding of the critical role of ECM in regulation of every cellular function.

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Key points

1. Thrombospondins are regulators of local inflammation and atherogenesis.
2. Thrombospondins are critical regulators of structure and function of myocardium.
3. Thrombospondins mediate the effects of aging, hypoxia and hyperglycemia in cardiovascular system.
4. Thrombospondins are important regulators of immunity.
5. Thrombospondins are important mediators of cellular homeostasis with numerous interactions inside the cells, on the cell surface and in ECM.

Figure 1. Thrombospondin protein family

TSP protein family includes 5 members: TSP-1, TSP-2, TSP-3, TSP-4 and TSP-5 (Cartilage Oligomeric Matrix Protein, COMP). The division in two subgroups is based on the domain structure (TSP-1 and TSP-2 have vWF domain and Type 1 TSP repeats that bear CD36 binding and MMP2 inhibition sites) and in the mode of oligomerization (TSP-1 and TSP-1 are trimers; TSP-3, TSP-4 and TSP-5 are pentamers). The large high homology C-terminal part of TSPs includes Type 2 EGF-like repeats, Type 3 Ca²⁺-binding repeats and the globular C-terminal domain. CD47 binding site is located in the globular C-terminal domain. NTD – N-terminal domain, specific for each TSP. CTD – globular C-terminal domain.

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Figure 2. Function of TSP-1 and TSP-4 in atherosclerotic lesion

Both TSP-1 and TSP-4 affect lesions content without affecting lipoprotein accumulation in the lesion at the later inflammatory stages.

TSP-4 is required for macrophage recruitment into the lesion and their migration through the lesion: fewer macrophages are found in *Thbs4*^{-/-}/*ApoE*^{-/-} mice, and they are accumulating close to the surface (A). This results in lower local inflammation (lower levels of adhesion proteins and cytokines).

TSP-1 regulates phagocytosis in macrophages (B). Lesions of *Thbs4*^{-/-}/*ApoE*^{-/-} mice are more inflammatory and have larger necrotic core as a result of insufficient phagocytosis.

Table 1

	TSP-1	TSP-2	TSP-3	TSP-4	TSP-5
Subfamily	A	A	B	B	B
Regulates angiogenesis in myocardium [9, 31, 38*, 42]	Inhibits	Promotes	Unknown	Unknown	Unknown
Expression in vascular wall [17, 50]	Yes	Yes	Yes	Yes	Yes
Increased expression in remodeling myocardium [9, 27-37, 46, 47]	Yes	Yes	Yes	Yes	Yes
Regulates fibrosis in myocardium [28*, 31, 32, 35, 37- 39*, 40- 42]	Upregulates	Upregulates	Unknown	Downregulates	Unknown
Inhibits MMP3 in myocardium [39*, 42, 43]	Yes	Yes	No	No	No
Direct effect on cardiomyocyte or fibroblast function [1**, 28*, 37]	Yes	Unknown	Unknown	Yes	Unknown
Accelerates atherosclerotic lesion development [17, 50]	Yes	Unknown	Unknown	Yes	Unknown
Effect on macrophages in lesion [17, 50]	Promotes phagocytosis	Unknown	Unknown	Promotes recruitment (retention?)	Unknown
Effect on local vascular inflammation [17, 50]	Inhibits	Unknown	Unknown	Promotes	Unknown
Associated with diabetes/insulin resistance and vascular complications [40, 69*, 74 - 82]	Yes	Unknown	Unknown	Unknown	Unknown
Effects associated with aging [28*, 44*, 66*, 84**, - 86*, 88*]	Yes	Yes	Unknown	Yes	Unknown
Effects on immune cells [17, 50, 51, 89*, 90 - 98**]	Yes	Yes	Unknown	Yes	Unknown