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Review

TGF-β signaling in vascular fibrosis

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Abstract

Transforming growth factor-β (TGF-β) participates in the pathogenesis of multiple cardiovascular diseases, including hypertension, restenosis, atherosclerosis, cardiac hypertrophy and heart failure. TGF-β exerts pleiotropic effects on cardiovascular cells, regulating cell growth, fibrosis and inflammation. TGF-β has long been believed to be the most important extracellular matrix regulator. We review the complex mechanisms involved in TGF-β-mediated vascular fibrosis that includes the Smad signaling pathway, activation of protein kinases and crosstalk between these pathways. TGF-β blockade diminishes fibrosis in experimental models, however better antifibrotic targets are needed for an effective therapy in human fibrotic diseases. A good candidate is connective tissue growth factor (CTGF), a downstream mediator of TGF-β-induced fibrosis. Among the different factors involved in vascular fibrosis, Angiotensin II (AngII) has special interest. AngII can activate the Smad pathway independent of TGF-β and shares with TGF-β many intracellular signals implicated in fibrosis. Blockers of AngII have demonstrated beneficial effects on many cardiovascular diseases and are now one of the best options to block TGF-β fibrotic responses. A better knowledge of the intracellular signals of TGF-β can provide novel therapeutic approaches for fibrotic diseases.

Keywords: Angiotensin; Fibrosis; Smooth muscle; MAPK kinases; Signal transduction

1. Introduction

Transforming growth factor-β (TGF-β) superfamily consists of more than 40 members including TGF-β, activins, inhibins, growth differentiation factors and bone morphogenetic proteins (BMPs). All members of this family share common sequence elements and structural motifs. They are multifunctional regulators of cell division, differentiation, migration, adhesion, organization and death, promoting extracellular matrix (ECM) production, tissue homeostasis and embryogenesis [1–3].

Among these proteins, TGF-β has a crucial role in tissue homeostasis and the disruption of the TGF-β pathway has been implicated in many human diseases, including cancer, autoimmune, fibrotic, and cardiovascular diseases (Fig. 1).

Three different TGF-β isoforms have been described, TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is the most important isoform for the cardiovascular system, and is present in endothelial cells, vascular smooth muscle cells (VSMC), myofibroblasts, macrophages and other hematopoietic cells [4]. TGF-β synthesis is a complex process (Fig. 2), extensively reviewed elsewhere [4]. TGF-β is synthesized as an inactive protein, named latent TGF-β, that consists of a main region and a latency associated peptide (LAP). This protein interacts with the latent TGF-β binding proteins (LTBP) and is anchored in the ECM. TGF-β is activated by proteolytic cleavage, thrombospondin-1 (Tsp-1), plasmin, acidic microenvironments, matrix metalloproteinases (MMP-2 and -9), and β6 integrin [4–6]. Several factors involved in cardiovascular damage regulate TGF-β synthesis and activation (Fig. 2). In VSMC, angiotensin II (AngII) stimulates TGF-β mRNA expression and promotes its conversion to the biologically active form [7]. AngII enhances TGF-β expression by transcriptional and posttranscriptional dependent mechanisms. *In vitro* studies have

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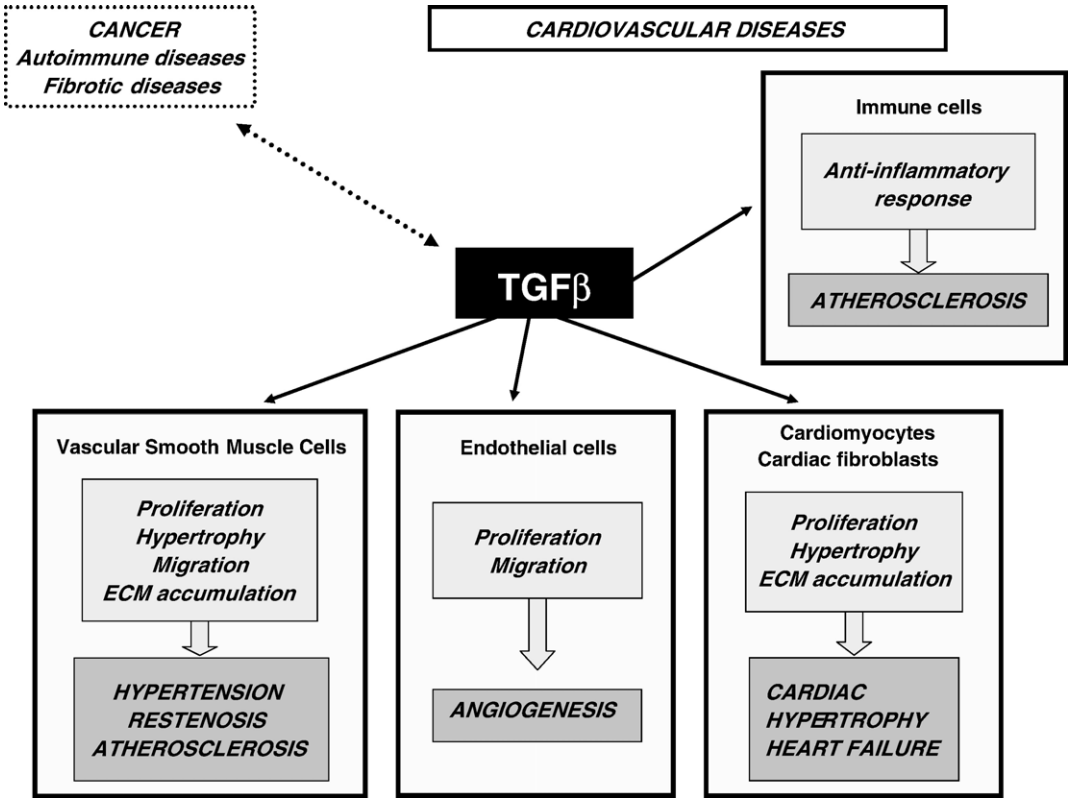

Fig. 1. TGF-β participates in many human diseases, including cancer, autoimmune diseases and fibrotic disorders as well as cardiovascular diseases. TGF-β regulates cellular responses in a positive or negative way. For example, the anti-inflammatory properties of TGF-β are beneficial in atherosclerosis, while the profibrotic effects contribute to fibrosis in hypertension and cardiac damage. For more details see text.

shown that AngII, through PKC and p38 MAPK-dependent pathways, activates the binding of nuclear proteins to the binding site of the activator protein-1 (AP-1) of the TGF-β1 promoter and stimulates transcriptional activity [8]. In VSMC MMP-2 enhances active TGF-β1. Active TGF-β, its receptors and receptor-mediated signaling are increased within the aortic wall with aging [9]. In mesangial cells oxidized low-density lipoprotein increases TGF-β and induces Smad-mediated activation of plasminogen activator inhibitor-1 (PAI-1) transcription [10]. Some of these agents can also enhance the concentration of the TGF-β1 protein by additional mechanisms. AngII and endothelin-1 stimulate Tsp-1 that, in turn, leads to an increased release of active TGF-β1 from the inactive latent complex [5].

The intensive research in the TGF-β field in the last years has provided important information about the complexity of TGF-β signaling pathways that lead to the many different responses, as reviewed by Massague et al. [1]. In the present paper, we discuss the importance of TGF-β in cardiovascular pathology, describing the signaling systems and the interactions involved in cellular responses.

2. TGF-β in cardiovascular pathology

TGF-β participates in the pathogenesis of many cardiovascular diseases, including hypertension, restenosis, atherosclerosis, cardiac hypertrophy, and heart failure (Fig. 1). TGF-β exerts pleiotropic effects on cardiovascular cells. This growth factor can regulate in a positive or negative way systems involved in cell proliferation, apoptosis, differentiation and migration. In cultured VSMC, TGF-β has a dual effect on cell growth. At low concentrations (<0.1 ng/ml) TGF-β is growth promoting, while at higher concentrations it is growth inhibiting, probably due to modulation of PDGF-A and PDGF-B receptor levels [11]. The complexity of TGF-β growth effects is enlarged when combinations of growth factors are examined (*e.g.*, AngII). Gibbons and colleagues [6] observed that when TGF-β was blocked AngII stimulated DNA synthesis and cell division of VSMC from normotensive rats, suggesting that AngII stimulates hyperplasia when PDGF-A is the dominantly expressed growth factor, whereas AngII stimulates cell hypertrophy when TGF-β is dominantly expressed.

The regulation of differentiation and cell growth in VSMC is critical to prevent proliferation associated with vascular damage. Under normal conditions VSMC in the arterial wall exhibit a contractile phenotype. However, VSMC retain the ability to de-differentiate into a proliferative phenotype, which is involved in vascular diseases, such as atherosclerosis and restenosis. Increased TGF-β in the arterial wall has been associated with neointima formation in chronically rejecting renal allografts [12]. In experimental

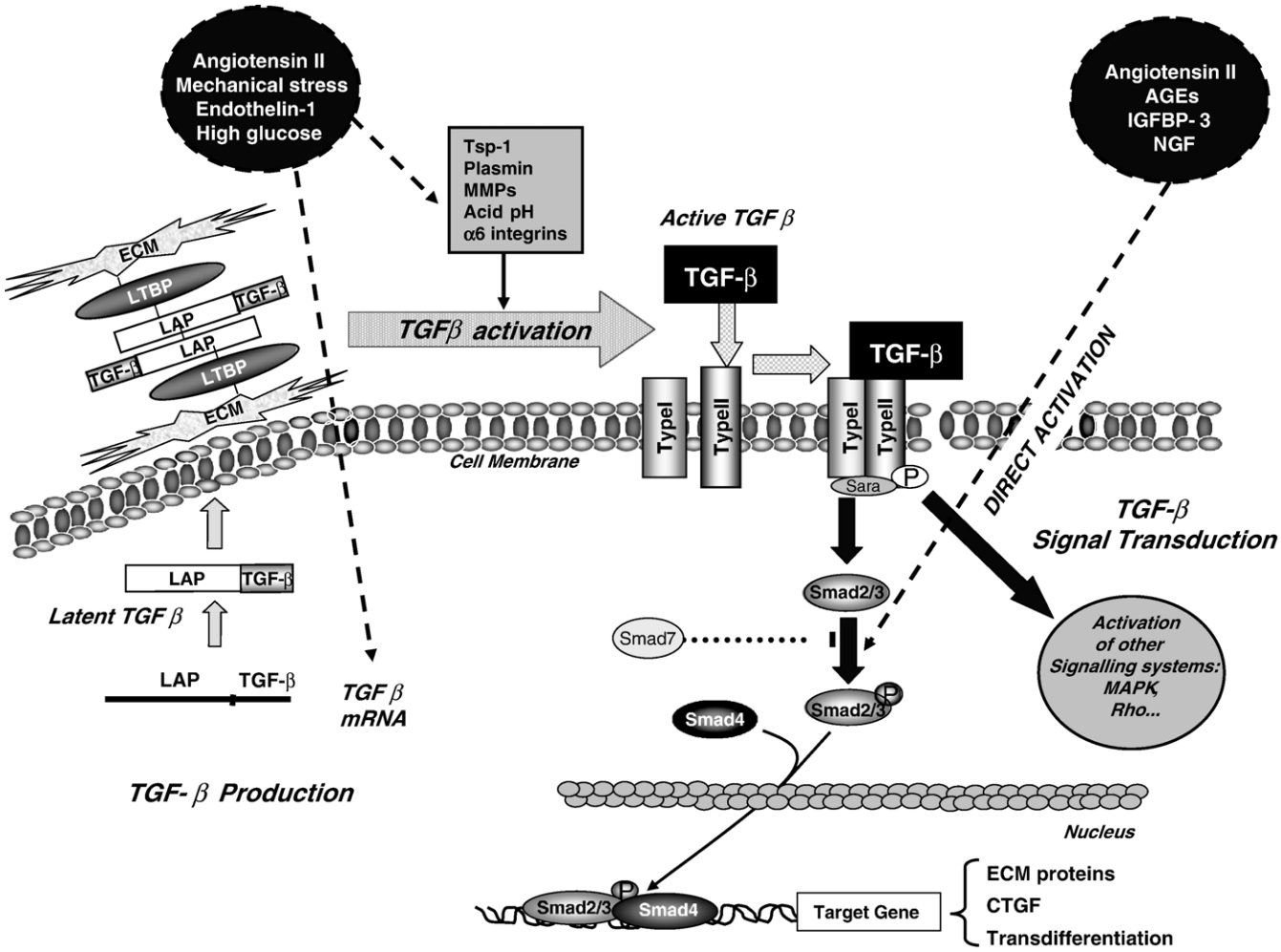

Fig. 2. TGF-β synthesis and signalling system. Several factors involved in cardiovascular damage (Angiotensin II, mechanical stress and high glucose) increase TGF-β mRNA expression and protein production. TGF-β is produced as a latent protein that is activated by TSP-1 and plasmin, among others. Active TGF-β binds to its receptors and activates the Smad pathway, which regulates the transcription of several genes involved in fibrosis, including CTGF. Besides direct activation of Smad by TGF-β, other factors can activate Smad pathway independently of TGF-β. TGF-β also elicits other intracellular signals. For more details see text.

models targeting TGF-β prevents neointima formation and the constrictive remodeling associated with angioplasty [13].

Other important characteristics of TGF-β are its anti-inflammatory and profibrotic properties. In atherosclerosis TGF-β has been considered as a "protective cytokine" [14] as it plays an important role in maintaining normal vessel wall structure and controls the balance between inflammation and ECM deposition. The loss of this protective effect, attributed to changes in TGF-β receptor profiles and modulated by local levels of TGF-β, contributes to the development of atherosclerosis. Studies done by Graiger et al support this hypothesis [14]. In the normal vessel, type II receptor is the most abundant. TGF-β through this receptor increases contractile protein expression, but not ECM production. In the diseased vessel, type I receptor is upregulated, and then TGF-β stimulates ECM production and could promote early fatty streak lesion formation [14]. In experimental models the lack of TGF-β1 signaling promotes the development of atherosclerotic lesions and unstable plaques. TGF-β has protective anti-inflammatory properties due to its immunomodulating effects on cells important in atherosclerotic lesion formation, including endothelial cells, VSMC, macrophages, and T cells. Finally, TGF-β can stimulate VSMC to produce collagen, therefore contributing to plaque stabilization. The role of TGF-β in atherosclerosis is extensively reviewed in this issue [15].

In contrast, cardiac fibrosis is thought to be partially mediated by TGF-β1, a potent stimulator of collagen-producing cardiac fibroblasts [16,17]. TGF-β participates in a multitude of heart diseases, including dilated and hypertrophic cardiomyopathies, post-infarction myocardial remodeling, valvular diseases and arrhythmias [16,17].

Hypertension causes structural changes in the arteries, including hypertrophy of VSMC, collagen and fibronectin accumulation, and destruction of elastic fibers. ECM overproduction has been attributed to hemodynamic changes associated with mechanical stress as well as to growth factors, such as TGF-β. Recent evidence shows that Emilin1

(an inhibitor of active TGF-β formation) knockout mice have increased blood pressure, peripheral vasculature resistance, and reduced vessel size indicating a potential role for TGF-β in hypertension [18].

The relationship between TGF-β and aortic aneurysm has special interest, but it is still controversial. TGF-β1 overexpression by endovascular gene therapy stabilizes abdominal aortic aneurysms already affected by inflammation and proteolysis [19]. Mutations in the genes encoding TGF-β receptors predispose to aggressive and widespread vascular disease, as noted in patients with Loeys-Dietz syndrome, an autosomal dominant aortic-aneurysm syndrome [20]. As it is well known, aortic aneurysm and dissection are manifestations of Marfan syndrome, a disorder caused by mutations in fibrillin-1 gene. In a mouse model of Marfan syndrome, aortic aneurysm was associated with increased TGF-β signaling and can be prevented by TGF-β antagonists, such as TGF-β-neutralizing antibody or the angiotensin II type 1 receptor (AT1) blocker, losartan [21].

3. TGF-β and vascular fibrosis

TGF-β has long been believed to be the most important ECM regulator [5]. In VSMC, endothelial cells, and fibroblasts, TGF-β1 increases the synthesis of ECM proteins, such as fibronectin, collagens and PAI-1, even at low concentrations [5,22]. TGF-β induces expression of the ED-A form of fibronectin which is required for enhancement of α-smooth muscle actin (α-SMA) and collagen type I expression [5]. PAI-1 is a serpin class protease inhibitor, important in tissue remodeling by modulating thrombosis, inflammation, migration, and ECM. TGF-β reduces collagenase production and stimulates the expression of tissue inhibitor of metalloproteinases (TIMP), resulting in an overall inhibition of ECM degradation and leading to excessive matrix accumulation [5,22]. The mechanisms involved in TGF-β-mediated vascular fibrosis are complex, including activation of Smad proteins, protein kinases, production of mediators and crosstalk between pathways, as we discuss here in detail. On the other hand, TGF-β also acts as a mediator of vascular fibrosis induced by several agents involved in cardiovascular diseases, including mechanical stress, AngII, high glucose, and advanced glycation products (AGEs) [23–25]. At the moment, there is no effective therapy for fibrotic diseases. The knowledge of molecular mechanisms involved in ECM accumulation may contribute to a better understanding of this pathological process and improve therapeutic strategies.

4. TGF-β receptors

There are two main TGF-β receptors: TGF-β receptor type I (TRI) and TGF-β receptor type II (TRII). Both are transmembrane receptors with serin–threonin kinase activity. TGF-β binds to TRII, inducing a change in this receptor which allows dimerization with TRI and its phosphorylation. Then, this active complex transmits TGF-β signaling into the cell [1–3,26].

TRI is also known as activin like kinase (ALK). Seven ALKs have been described in mammals [26]. ALKs have been implicated in several disorders, including tumorigenesis, hemorrhagic telangiectasia (HHT), immune and renal diseases, and skeletal malfunctions, suggesting that these receptors can be used as drug targets [27]. The main ALKs in the vascular system are ALK1 and ALK5, differing in the used signalling pathway. ALK1 activates Smad1/5, while ALK5 activates Smad2/3. This has recently been associated with a model in which, depending on the ALK used, endothelial cells respond oppositely to TGF-β, playing a balancing role for controlling the proliferation and migration of endothelial cells during angiogenesis. ALK1, *via* Smad1/5, stimulates endothelial cells proliferation and migration, whereas ALK5 *via* Smad2/3 inhibits these processes [28]. Studies using ALK1 and ALK5-null mice reveal distinct roles in vascular development. ALK1-null embryos present severe dilation of the vascular lumens, while ALK5-null embryos exhibit a defect in the formation of VSMC layers [29]. In blood vessels, ALK1 is expressed in the arterial endothelium and ALK5 is localized in the medial and adventitial layers of blood vessels, but is undetectable in the intimal layer. In vascular cells, including endothelial cells and VSMC, the more abundant receptor is ALK5, with ALK5/Smad2/3 signalling pathway being the most frequently used.

Another type of accessory TGF-β receptors, “the type III receptor”, which includes endoglin and betaglycan, is a very controversial group because of its heterogeneity, They do not have signal transduction capacity although can be regulated by the other TGF-β receptors. The function of endoglin in vascular responses is not clear, however it is known to capture soluble TGF-β thereby helping TRII in its recruitment [30,31]. Betaglycan can act as an inhibitor or an enhancer of TGF-β responses [32].

5. TGF-β signaling systems: the Smad pathway

TGF-β predominantly transmits the signals through cytoplasmic proteins called Smads, which translocate into the cell nucleus acting as transcription factors [1,2]. Eight different members of the Smad family have been identified in mammals. Based on their function, the Smads are classified as receptor-activated (R-) Smads (Smad1, -2,-3, -5 and -8), common-partner (Co-) Smads (Smad4) or inhibitory (I-) Smads (Smad6 and -7). Smad2 and Smad3 are specific mediators of TGF-β/activin pathways, whereas Smad1, Smad5 and Smad8 are involved in BMP signaling [1,2]. Smad4 forms hetero-oligomers with R-Smads and is a shared mediator of TGF-β and BMP signaling. Smad4 continuously shuttles between cytoplasm and nucleus. Its cytoplasmic localization in unstimulated cells is due to active nuclear export. Inactive R-Smads reside predominantly in the cytoplasm whereas the I-Smads localize to the cell

nucleus. Smad6 and Smad7 act as major negative regulators forming autoinhibitory feedback loops and mediate the cross-talking with other signaling pathways. Inhibitory Smads block TGF-β superfamily signalling by binding to the type I receptors (Smad7) or by competing with activated R-Smad1 for binding to Co-Smad4 (Smad6). At physiological concentrations, Smad6 may selectively inhibit BMP receptor signaling, whereas Smad7 inhibits both BMP and TGF-β/activin receptor signaling [1,2,33].

Upon activation TGF-β transduces its signal across the plasma membrane by binding to its specific receptors, as described earlier [1–3]. After receptor complex formation, they are internalized *via* clathrin coated pits into early endosomes that contain an accessory protein named SARA (Smad anchor for receptor activation). SARA is a FYVE domain containing scaffolding protein that interacts with the MH2 domain of inactive R-Smads, Smad2 and Smad3, targeting them to early endosomes and aiding in the recruitment of Smads, thus promoting Smad phosphorylation, at C-terminal serines [1–3]. The R-Smads then dissociate from the receptor complex to form oligomers at different stochiometries; heterotrimers with two R-Smads and one Smad4 (Smad3) or heterodimers consisting of an R-Smad (Smad2) and a Co-Smad. These complexes translocate to the nucleus and function as transcriptional regulators of target genes in a cell type specific manner through interactions with other transcription factors, corepressors and coactivators. Diverse ligand responses in different cell types are a result of different Smad-interacting transcription factors and of cooperation with other signaling pathways. The inhibitory Smad7 binds to activated type I receptor, thereby preventing phosphorylation of Smad2/3, or recruits the ubiquitine ligases Smurf1 and Smurf2 to induce proteasomal degradation of the receptor complexes [1–3].

Smad proteins are expressed in VSMC and mediate TGF-β signaling [34]. In VSMC, TGF-β1 increases phosphorylation of Smad2 and Smad3, that form heterotrimers with Smad4. This complex translocates into the nucleus, binds to Smad-related DNA sequences and increases the transcription of genes involved in vascular fibrosis such as fibronectin, type I collagen and connective tissue growth factor (CTGF) (Fig. 2) [34–36]. In these cells, overexpression of Smad7 inhibites TGF-β1-induced ECM and CTGF expression [35,36].

The expression levels of Smad proteins can profoundly affect signaling. The level of Smad proteins can be controlled by interactions with various components of the 26S proteasome system [37]. The E3 ligases, Smurf1 and -2, as well as SCF/Roc1, antagonize TGF-β family signaling through interaction with R-Smads, thereby targeting them for degradation and terminating Smad-mediated signaling [1–3]. Phosphorylation of Smads induces their activation and modulates their activity. Smads can be activated by phosphorylation *via* MAPK/ERK and Ca2+/calmodulin-dependent protein kinase II pathways [1–3]. Induction of Smad6 and Smad7 expression by BMP and TGF-β respectively, represents an auto-inhibitory feedback mechanism for ligand-induced signaling.

Several agents involved in vascular damage also regulate Smad protein expression levels. Activation of the epidermal growth factor (EGF) receptor, interferon-γ signalling through STAT (signal transducer and activator of transcription) proteins, and activation of NF-κB by tumour-necrosis factor-α, induce Smad7 expression [9,14]. In VSMC AngII increases protein levels of Smad2 and Smad4 *in vivo* and *in vitro* [34]. Smad6 overexpression correlates with increased TGF-β and PAI-1 levels found in diseased vessels [38]. Elevated Smad expression has been described in different pathological conditions, including hypertension, atherosclerosis, and cardiac damage [5,17,35,36].

Besides regulation of Smad levels, TGF-β pathway can be blocked by ligands,-extracellular ligand trapping molecules or antagonists-, such as gremlin, noggin, chordin (all members of DAN/Cerberus protein family), and follistatin, which bind to the receptors. Another antagonist is the naturally occurring pseudoreceptor BAMBI (BMP and Activin membrane bound inhibitor) which extracellularly resembles a type I receptor but lacks the cytosolic kinase domain. BAMBI can form stable associations with various TGF-β family type I receptors thus blocking BMP, Activin and TGF-β signalling [39].

The dysregulation of TGF-β/Smad signaling has been implicated in the pathogenesis of human diseases. Expression of I-Smads is mainly regulated at transcriptional level and by post-translational protein degradation and their intracellular levels are tightly controlled to maintain the homeostatic balances. However, abnormal levels of I-Smads in pathological conditions elicit the altered TGF-β signaling in cells, eventually causing TGF-β-related human diseases [1–3].

6. Smad and vascular fibrosis

TGF-β, *via* Smad activation, upregulates the transcription of several genes important for ECM formation, such as procollagens, fibronectin, CTGF and PAI-1 [35,40–42]. Overexpression of some Smad proteins activates transcription of some of these genes, like PAI-1, even in the absence of TGF-β [1–3]. In VSMC, overexpression of Smad7 inhibits TGF-β-induced fibronectin, collagen and CTGF expression [35], and ectopic expression of Smad3 stimulates TGF-β-induced fibronectin synthesis [43], demonstrating the important role of Smad signaling in vascular fibrosis.

Adventitial cells contribute to constrictive remodeling after vascular injury by migrating to the neointima and synthesizing EMC. Smad7 overexpression in adventitial cells attenuates collagen deposition, remodeling and contribution of adventitial fibroblasts to neointima formation after balloon angioplasty, showing a novel target for gene therapy to reduce the incidence of restenosis after angioplasty [44]. Several works have demonstrated that Smad7 gene transfer blocks fibrosis in other cell systems and diseases [5].

There are fundamental differences in the processes mediated by Smad2 and Smad3 *in vivo*. Mice homozygous for a deletion in Smad2 die during embryogenesis [45], suggesting a role in normal development, whereas mice homozygous for a deletion of Smad3 are viable and fertile [46]. In adult fibroblasts Smad3 is required for TGF-β-induced gene expression [47], indicating that Smad3 plays an important role in fibrosis. Studies of the vascular response to injury in Smad3-null mice showed that these animals presented enhancement of neointimal hyperplasia, and more proliferating VSMC with a less amount of collagen compared with wild-type intima [48], suggesting a vasculoprotective role of endogenous Smad3 in response to injury.

Activation of Smad pathway has been described in several diseases associated with fibrosis [9,14]. In a model of hypertension-induced vascular damage, aortic Smad2 phosphorylation correlated with an upregulation of ECM proteins and profibrotic factors were found [35]. In human atheroma plaques, VSMC within fibrofatty lesions did not express the Smad proteins, while Smad2, Smad3, and Smad4 are expressed in VSMC of fibrous plaques and macrophages in fatty streaks/fibrofatty lesions [49]. The Smad pathway can be activated in infiltrating cells, inducing anti-inflammatory responses, and in VSMC thereby increasing ECM production. The lack of key TGF-β signaling components in VSMC of fibrofatty lesions indicates impaired ability of these cells to initiate TGF-β-mediated Smad-dependent transcriptional responses. These data support the hypothesis of the beneficial effect of TGF-β in atherosclerosis.

The role of Smad proteins in cardiac functions has been recently reviewed [17,18], showing that BMP participates in heart development, and activation of Smads play an important role in cardiac remodeling and heart failure, through the regulation of fibrotic, apoptotic, and anti-hypertrophic processes.

7. Other TGF-β signaling systems involved in vascular fibrosis

Growing biochemical and developmental evidence supports the notion that alternative, non-Smad pathways also participate in TGF-β signalling and serve as nodes for crosstalk with other major signaling pathways [reviewed in [3]].

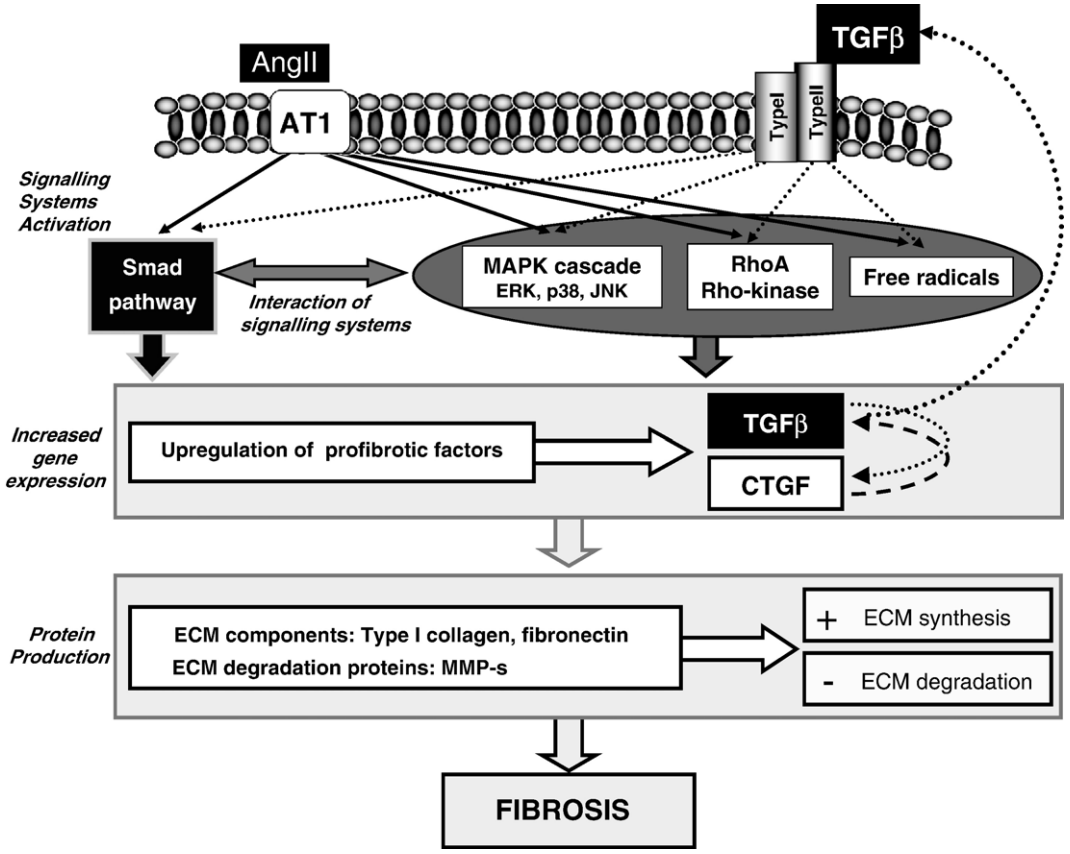

Fig. 3. Molecular mechanisms implicated in vascular fibrosis caused by AngII and TGF-β. AngII activates several intracellular signalling systems common to TGF-β, including activation of the Smad pathway, protein kinases (MAPK and Rho-kinase) and production of reactive oxygen species (ROS), which regulate the expression of profibrotic mediators (CTGF) and ECM turnover (by increasing the synthesis of ECM proteins and inhibiting their degradation). CTGF is an important profibrotic mediator that acts as a downstream mediator of TGF-β and AngII-induced ECM production. Moreover, CTGF increases TGF-β responses and both factors synergize to promote persistent fibrosis.

7.1. The mitogen-activated protein kinase (MAPK) pathway

Members of the mitogen-activated protein kinase (MAPK) family are frequently involved in TGF-β/Smad signaling. In VSMC, TGF-β activates the three MAPK: extracellular signal-regulated kinases (ERK), p38 MAPK, and c-*Jun* N-terminal kinases (JNK) (Fig. 3). There is a cross-talk between ERK and Smad [50]. Nuclear accumulation of activated Smads can be modulated by Ras activated ERK kinases. Epidermal growth factor (EGF), hepatocyte growth factor, oncogenic Ras and AngII stimulate ERK kinases, which in turn phosphorylate Smad proteins [1–3]. ERK phosphorylates serine residues in the linker regions of Smad1, Smad2 and Smad3. ERK inhibition reduces TGF-β-stimulated Smad phosphorylation as well as collagen production and promoter activities, suggesting that ERK activity is necessary for an optimal response to TGF-β [50]. Overexpression of constitutively active members of the ras/MEK/ERK cascade promotes Smad3-dependent processes in kidney mesangial cells but blocks nuclear accumulation of Smads in epithelial cells [5], showing clear differences between cell types. Other MAPK-mediated TGF-β responses are independent of Smads. TGF-β leads to potent growth inhibition through G0/G1 arrest, that is specifically attenuated by pharmacological blockade of p38 MAPK, but not of p42/44 or JNK pathway, without alteration of Smad function [51]. In VSMC, TGF-β upregulates PAI-1 *via* src-kinase, MEK signaling and EGF-receptor transactivation [52]. However, the interactions between TGF-β and MAPK signaling remain to be elucidated *in vivo* in the vasculature.

7.2. The small G proteins

The Rho family of GTP-binding proteins includes Rho, Rac, and Cdc42 proteins and play an important role in cell adhesion, actin dynamics and regulation of gene transcription [53]. RhoA is a modulator of Smad activation. Transfection with a dominant negative RhoA blocked phosphorylation and nuclear translocation of Smad2 and Smad3 caused by TGF-β1 [55]. RhoA and p38 MAPK participate in TGF-β1 induced VSMC differentiation, through the activation of serum-response factor (SRF), GATA, and MEF2-dependent enhancer-reporters [54]. DeltaEF1 transcription factor is upregulated during VSMC differentiation and selectively transactivates the promoters of VSMC differentiation marker genes, such as α-SMA and SM myosin heavy chain. DeltaEF1 physically interacts with SRF and Smad3, resulting in a synergistic activation of the α-SMA promoter [56].

Several findings suggest a potential role for the Rho/Rho-kinase signalling pathway in the development of fibrotic lesions. Many works have demonstrated that Rho-kinase inhibitors diminish fibrosis in experimental models of vascular and renal damage. In some of these models Rho-kinase inhibition diminished gene overexpression of α-SMA, TGF-β and ECM proteins [57–59], suggesting that Rho-kinase inhibitors could be novel targets for fibrotic therapy.

7.3. Redox mechanisms

Reactive oxygen species (ROS) act as second messengers of TGF-β1 regulating a number of important cellular events, including fibrosis and atherogenesis [60–62]. TGF-β can induce ROS production in different cell types. Most of the O_2^- generated in VSMC appears to be produced by the intracellular NAD(P)H oxidase, which includes a novel p91 homolog termed Nox1. In human pulmonary artery smooth muscle cells, TGF-β1 regulates cell growth by a redox-dependent mechanism mediated through Nox4 induction [63]. Under pathological conditions elevated ROS concentration contribute to vascular dysfunction and remodeling through oxidative damage. Inhibition of NAD(P)H oxidase activity is now being considered, at least experimentally, as a possible therapeutic target in the treatment of hypertension. In fact it has been suggested that some of the beneficial actions of classical antihypertensive drugs may be mediated, in part, by decreasing vascular oxidative stress.

8. Relation between TGF-β and CTGF

Connective tissue growth factor (CTGF) has recently been described as a novel profibrotic factor that mediates some TGF-β responses, including apoptosis and fibrosis [64]. *In vivo*, blockade of CTGF synthesis or activity reduces TGF-β-induced collagen synthesis. Data suggests that CTGF and TGF-β synergize to promote chronic fibrosis. In mice, subcutaneous co-injection of CTGF and TGF-β results in sustained and persistent fibrosis. CTGF is upregulated in a variety of fibrotic disorders, in the skin, kidney, lung and cardiovascular system [64]. CTGF overexpression associated with ECM accumulation has been described in human atherosclerotic lesions, after myocardial infarction and in vascular and cardiac tissues in experimental hypertension [65–68]. Depending on the cell type, CTGF has diverse bioactivities, including regulation of proliferation/apoptosis, induction of chemotaxis, cellular adhesion, ECM production and angiogenesis [64]. In VSMC CTGF regulates cell proliferation/apoptosis, migration and ECM synthesis [69,70]. In these cells, different factors involved in vascular damage, including cyclic mechanical stretching, elevated glucose concentrations, AngII, ET-1, and TGF-β upregulate not only CTGF but also ECM proteins which can be reversed by blocking endogenous CTGF production [68,70], showing that CTGF is a downstream fibrotic mediator.

CTGF has been described to bind directly to TGF-β. This binding leads to an enhancement of TGF-β activity. The mechanism is based on a chaperon function of CTGF, which increases the affinity of TGF-β to its different receptors, hence their response is more intense and more prolonged [71]. This is not the only way by which CTGF enhances

TGF-β responses. Endogenous production of CTGF by TGF-β leads to Smad7 transcriptional suppression, *via* induction of the transcription factor TIEG-1. By this mechanisms CTGF blocks the negative feedback loop provided by Smad7, perpetuating the activation of TGF-β signaling [72]. This could be relevant in pathological conditions where CTGF is highly upregulated.

Much evidence shows that TGF-β participates in the fibrotic process *in vivo*. The blockade of TGF-β activity with neutralizing antibodies and decorin, a scavenger of its active form, has demonstrated reduction of fibrosis in experimental models of acute injury; however, the TGF-β knockout mice is lethal, displaying severely impaired late-stage wound repair, including collagen deposition, and presents a hyperinflammatory phenotype [73], suggesting that more specific targets for antifibrotic therapy are necessary. Mice heterozygous for CTGF gene deletion present defects in matrix organization and synthesis during osteogenesis, resulting in a major defect in the development of the skeletal component of the rib cage and consequently die immediately after birth [74]. Since there is no data regarding positive effects of CTGF in the modulation of inflammatory and immune reactions, and this growth factor is a downstream mediator of fibrosis caused by TGF-β and other factors involved in tissue injury, CTGF maybe a useful target for novel antifibrotic therapies.

9. Relation between AngII and TGF-β

Besides TGF-β, ECM accumulation has been attributed to different factors involved in vascular damage, such as AngII, high glucose concentrations as well as to hemodynamic changes associated with mechanical stress [23].

The interrelation between AngII and TGF-β is already established. In different pathological settings and cell types, AngII regulates TGF-β expression and activation, and the endogenous production of TGF-β mediates some AngII responses [23,75]. Angiotensin antagonism attenuated TGF-β secretion and fibrosis. AngII-induced ECM synthesis was inhibited by a TGF-β neutralizing antibody or truncated TGF-β type II receptor, suggesting a linear pathway where AngII stimulates TGF-β secretion, which in turn triggers ECM synthesis [23,75]. AngII and TGF-β share some intracellular mechanisms involved in fibrosis, including activation of protein kinases, production of growth factors, and activation of the Smad pathway (Fig. 3).

9.1. Smad pathway and vascular fibrosis caused by AngII

We have recently shown that in VSMC, AngII caused a rapid Smad2 phosphorylation, nuclear translocation of phosphorylated-Smad2 and Smad4, and increased Smad DNA-binding activity, with a maximal response after 20 min of stimulation [35]. AngII-mediated Smad activation was independent of endogenous TGF-β production or activation, demonstrated by blocking TGF-β with a neutralizing antibody against active TGF-β, and decorin [35]. The studies performed by Wang et al. *via* overexpression of a dominant negative TGF-β receptor II or conditional deletion of this receptor have confirmed this observation [36]. Some evidence suggests that AngII activates the Smad pathway in other cells. In cultured primary cardiac rat fibroblasts, AngII caused rapid phosphorylated-Smad2 nuclear translocation [76]. In tubuloepithelial cells the early Smad activation caused by AngII was TGF-β independent. However, AngII-induced long-term Smad activation and the transdifferentiation of renal tubuloepithelial cells to myofibroblasts were mediated by TGF-β [77].

As commented above, the Smad mediated gene transcription is subject to regulation by other signaling pathways, such as MAPK. Many AngII responses are mediated by MAPK, including the regulation of profibrotic factors and ECM proteins [23]. We have found that the p38 MAPK inhibitor SB203580 diminished AngII-induced Smad2 phosphorylation [35]. In the mouse primary aorta an ERK1/2 inhibitor and transfection with a dominant-negative ERK1/2 inhibited AngII-induced Smad signaling [36]. These data show that AngII activates Smad pathway, *via* MAPK activation, independently of TGF-β (Fig. 3).

Several *in vivo* data suggest that AngII activates the Smad pathway. The blockade of AngII type I receptors diminishes Smad pathway activation in myocardial infarction in rats, in hypertension-induced vascular damage and in an experimental model of renal damage [76,35,78]. Rats infused with AngII for 3 days presented activated Smad signaling in the aorta, observed by positive staining for phosphorylated-Smad2 mainly found in the nuclei of VSMC, which was not detected in control animals [35]. Smad overexpression was correlated to CTGF induction, preceding the accumulation of ECM proteins observed after 7 days, suggesting that Smad participates in the progression of vascular fibrosis. In cultured VSMC we have observed that Smad7 overexpression decreased CTGF, fibronectin and type1 procollagen upregulation caused by AngII. All this data suggest that the Smad pathway could contribute to the profibrogenic effects of AngII in vascular diseases.

9.2. Other signaling systems and mediators common to AngII and TGF-β

CTGF is a downstream mediator of AngII and TGF-β-induced ECM production [64,68]. The mechanisms involved in its regulation also present some similarities. CTGF induction by TGF-β and AngII in mesangial cells and fibroblasts requires the ras/MEK/ERK cascade [79]. In VSMC CTGF regulation by both factors is mediated by the Smad pathway, MAPK, and RhoA/Rho-kinase activation, and redox mechanisms [35,79]. AngII, *via* NAD(P)H oxidase/ROS production, regulates vascular responses, including hypertrophy and fibrosis [80]. Angiotensin blockers have demonstrated beneficial effect in many cardiovascular diseases [23,79]. These drugs interfere with TGF-β

synthesis and block activation of the Smad pathway, showing that they are good candidates for the blockade of TGF-β activities.

10. TGF-β-independent Smad activation

Novel data has demonstrated that several factors involved in cardiovascular damage can activate the Smad signaling pathway, independently of endogenous synthesis of TGF-β (Fig. 2). In cells transfected with theTGF-β receptor type II, insulin-like growth factor binding protein-3 (IGFBP-3) stimulated Smad2/3 phosphorylation, potentiated TGF-β1-stimulated Smad phosphorylation, and cooperate with exogenous TGF-β1 in cell growth inhibition [81]. In PC12 cells, nerve growth factor caused activation of the Smad pathway independent of TGF-β [82]. Advanced glycation end products (AGEs) caused rapid Smad2/3 activation in renal and vascular cells that was independent of TGF-β [25]. After incubation with AGEs for 24 h TGF-β was produced and activated Smads by the classic pathway. AGE-induced long-term Smad activation and collagen synthesis was inhibited by ERK/p38 MAPK inhibitors, but not by TGF-β blockade, suggesting that the MAPK-Smad signaling crosstalk pathway is a key mechanism in diabetic organ injury. This data provides further evidence that the Smad proteins are not exclusively activated by the classical TGF-β triggered mechanism.

11. Conclusion

TGF-β participates in the fibrotic process, activating cells directly or acting as mediator of many important agents involved in cardiovascular damage, including mechanical stress, AngII and high glucose concentrations. Many studies have demonstrated that TGF-β blockade diminishes experimental fibrosis, however the search for better targets, such as CTGF, is necessary for the therapeutic approach to chronic fibrotic diseases. On the other hand, in atherosclerosis TGF-β has been suggested to be a protective cytokine due to its inhibitory action on T cells. However, the direct effects of TGF-β on VSMC could also have an impact on the development of human atherosclerotic lesions. Recent advances in cancer research in the molecular mechanisms implicated in the loss of the anti-tumor suppression properties of TGF-β during tumor progression [83], could give us some clues to understanding the complexity of TGF-β responses that varies between cell types and disease conditions.

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