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TGF- β and Restenosis Revisited: A Smad Link

Pasithorn A. Suwanabol, M.D., K. Craig Kent, M.D., and Bo Liu, Ph.D.¹

Department of Surgery, Division of Vascular Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin

Abstract

Despite novel surgical therapies for the treatment of atherosclerosis, restenosis continues to be a significant impediment to the long-term success of vascular interventions. Transforming growth factor-beta (TGF- β), a family of cytokines found to be up-regulated at sites of arterial injury, has long been implicated in restenosis; a role that has largely been attributed to TGF- β -mediated vascular fibrosis. However, emerging data indicate that the role of TGF- β in intimal thickening and arterial remodeling, the critical components of restenosis, is complex and multidirectional. Recent advancements have clarified the basic signaling pathway of TGF- β , making evident the need to redefine the precise role of this family of cytokines and its primary signaling pathway, Smad, in restenosis. Unraveling TGF- β signaling in intimal thickening and arterial remodeling will pave the way for a clearer understanding of restenosis and the development of innovative pharmacological therapies.

Keywords

restenosis; intimal hyperplasia; angioplasty; transforming growth factor-beta (TGF- β); Smad

THE HISTORY OF TGF- β AND RESTENOSIS

The role of transforming growth factor-beta (TGF- β) in restenosis has been studied for over two decades. TGF- β is a family of cytokines with a variety of functions including fibrosis, growth, differentiation, and apoptosis [1]. Emerging data from both *in vitro* and *in vivo* studies have demonstrated the importance of TGF- β in regulating the critical components of restenosis: intimal thickening and arterial remodeling.

TGF- β is Up-Regulated at Sites of Arterial Injury

Both human and animal studies demonstrate that TGF- β is up-regulated at sites of vascular injury (Table 1). Madri *et al.* first demonstrated the presence of TGF- β 1 in the neointima of rat carotid arteries by immunostaining 10 wk following balloon-catheter injury, and postulated that TGF- β may have a role in the synthesis of extracellular matrix (ECM) [2]. The source of TGF- β , however, was unknown at the time. Subsequently, Majesky *et al.* demonstrated in the same animal model that levels of TGF- β were increased by 6 h postinjury and that the majority of TGF- β was found within neointimal smooth muscle cells (SMC) [3].

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¹To whom correspondence and reprint requests should be addressed at Department of Surgery, University of Wisconsin School of Medicine and Public Health, 1111 Highland Avenue, WIMR 5120, Madison, WI 53705. liub@surgery.wisc.edu.

SUPPLEMENTARY DATA

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Nikol *et al.* then examined human specimens of restenotic lesions and discovered that TGF- β mRNA expression was highest in restenotic lesions compared with primary atherosclerotic lesions and nonatherosclerotic tissues. Additionally, the authors redemonstrated the source of TGF- β as vascular SMCs [4]. The presence of TGF- β in human restenotic lesions was confirmed by Yutani *et al.* Nine of 11 human restenotic lesions analyzed following percutaneous revascularization demonstrated the presence of TGF- β by immunohistochemistry [5].

More recently, Chamberlain *et al.* demonstrated in a porcine coronary artery model that active TGF- β peaks at 3 d following injury and returns to baseline at 28 d [6], which implicates a role of TGF- β in vascular injury in both peripheral and coronary arteries.

Up-Regulation of TGF- β Increases Intimal Thickening

Since the Nineties, several investigative groups have examined the role of TGF- β , specifically the TGF- β 1 isoform, by employing the gain of function and loss of function approaches in animal models of arterial injury (Table 1). Nabel *et al.* treated uninjured porcine arteries with an expression plasmid encoding active TGF- β 1 and demonstrated increased ECM production and cellular proliferation in the arterial wall [7]. These findings were supported by Kanzaki *et al.* who treated balloon-catheter-injured rabbit carotid arteries with 10 μ g/kg TGF- β and 10 mg/kg aspirin or 10 mg/kg aspirin only. The investigators found that the TGF- β 1-treated group exhibited significantly greater intimal thickening [8]. Wolff *et al.* treated rat vein grafts with adenovirus expressing TGF- β mRNA or mRNA antisense to TGF- β or empty adenovirus. Vein grafts treated with adenovirus expressing TGF- β mRNA exhibited a larger neointima, as measured by collagen content, compared with vein grafts treated with adenovirus expressing mRNA antisense to TGF- β and empty virus [9]. These studies demonstrate that up-regulation of TGF- β further stimulates intimal thickening.

Contrarily, a recent study by Kingston and colleagues demonstrate that gene transfer of TGF- β 1 causes a *decrease* in neointimal formation using a porcine coronary angioplasty model. The same authors, however, report enhanced neointimal formation when TGF- β 3 was overexpressed. The authors propose that in both injured vessels and healing cutaneous wounds, TGF- β 3 acts as an antagonist to TGF- β 1. Additionally, they propose that there is an inherent difference between coronary arteries and peripheral arteries [10].

Blockade of TGF- β Attenuates Intimal Thickening

In general, experimental strategies that inhibit TGF- β 1's function lead to a suppression of neointimal growth (Table 1). Wolf *et al.* treated rabbit balloon-catheter-injured carotid arteries with antibodies to TGF- β 1, control antibody or normal saline. Intravenous administration of anti-TGF- β 1 antibody was found to significantly reduce the size of the neointimal lesion [11]. Yamamoto and colleagues then confirmed the role of TGF- β by treating balloon-catheter-injured rat carotid arteries with ribozyme oligonucleotides against TGF- β . The investigators show that degradation of TGF- β 1 mRNA results in inhibition of neointimal formation [12].

Smith *et al.* treated rat balloon-catheter-injured carotid arteries with soluble TGF- β receptor II to inhibit the action of all three TGF- β isoforms and demonstrated a decrease in neointimal formation. The authors propose that this is the result of blockade of TGF- β -induced SMC and fibroblast proliferation [13]. The Kingston group subsequently treated porcine coronary arteries with adenovirus expressing a form of TGF- β type II receptor to antagonize the effects of TGF- β 1 following percutaneous transluminal coronary angioplasty (PTCA) and found that the arteries displayed less luminal loss and greater collagen

deposition compared with untreated arteries. However, the researchers suggest that rather than inhibiting SMC proliferation, the inhibition of TGF- β prevents constrictive remodeling; another critical component of restenosis. They propose that inhibition of TGF- β may decrease matrix metalloproteinase (MMP) expression, thereby reducing collagen degradation and subsequently creating an adventitial collagen scaffold to maintain vessel circumference [14].

TGF- β 's Role in Arterial Remodeling is Complex

Consequently, in recent years it has become recognized that recurrent disease following vascular reconstruction is related not only to intimal thickening but also the process of arterial remodeling. Arteries or vein grafts often respond to thickening of the intima in an adaptive manner by developing compensatory enlargement of the vessel wall. This increase in the overall diameter of the vessel, usually measured by an increase in the length of the external elastic lamina (EEL), prevents the thickened neointima from impinging on the lumen. Alternatively, when compensatory enlargement does not occur, or when the overall vessel circumference decreases, intimal thickening leads to narrowing of the vessel lumen; a process called constrictive remodeling [15–17].

While TGF- β is generally believed to stimulate intimal hyperplasia, its role in arterial remodeling remains unclear. Shi *et al.* treated porcine coronary arteries with TGF- β 1 following PTCA and demonstrated that TGF- β induces myofibroblast migration as well as arterial remodeling by collagen deposition [18]. As previously mentioned, Smith *et al.* treated rat carotid arteries with soluble TGF- β receptor inhibitor following balloon-catheter injury. In addition to a decrease in neointimal formation, the authors demonstrated an increase in EEL length suggesting that TGF- β induces constrictive remodeling [13]. This supports the aforementioned study performed by the Kingston group who found that blockade of TGF- β 1 decreases constrictive remodeling [14]. These authors performed a subsequent study investigating the role of two of three isoforms of TGF- β , - β 1 and - β 3, in restenosis. TGF- β 3 has been found to antagonize TGF- β 1's effect on ECM production both *in vitro* and *in vivo*. As a result, the investigators treated porcine coronary arteries with adenovirus expressing TGF- β 3 following PTCA and found that TGF- β 3-treated arteries led to less luminal area loss and increased external elastic lamina (EEL) and internal elastic lamina (IEL) areas compared with control injured and adenovirus expressing TGF- β 1-treated arteries. Although neointimal formation did not differ significantly between the control injured and TGF- β 3-treated groups, the TGF- β 3-treated arteries demonstrated increased total, adventitial, and medial plus neointimal collagen content. These findings suggest that TGF- β 3 decreases luminal loss following PTCA by inhibiting constrictive remodeling, which occurs, as proposed by the authors, through the creation of an adventitial collagen scaffold to prevent EEL and IEL area loss [10].

THE BASIC LOGIC OF TGF- β SIGNALING

TGF- β Ligands

The complicated roles of TGF- β in restenosis may result from the complex signaling network of this growth factor, a member of a superfamily consisting of a highly evolutionarily conserved family of growth factors including bone morphogenic proteins (BMP), growth differentiation factors (GDF), activins, TGF- β isoforms, nodals, and anti-mullerian hormone (AMH). The TGF- β cytokine consists of three isoforms: TGF- β 1, - β 2, and - β 3. The different isoforms within each cell type are not expressed uniformly. TGF- β 1 is expressed in epithelial cells, SMCs, hematopoietic cells, and fibroblasts. TGF- β 2, however, is localized on epithelial cells and neurons whereas TGF- β 3 is expressed primarily on

mesenchymal cells [19]. All three isoforms are found to be up-regulated in restenotic tissues [13].

The TGF- β ligands are synthesized within the cell as dimeric pro-hormones [20]. The latent TGF- β complex consists of TGF- β , a latency-associated peptide (LAP) and a latent TGF- β binding protein (LTBP) [21]. Latent dimeric forms are secreted into the ECM, where they are cleaved by furins and other convertases to form active signaling molecules when stimulated [13,22]. Angiotensin II, mechanical stress, endothelin-I, high glucose, extremes of temperature and pH, steroids, and reactive oxygen species have been found to stimulate TGF- β activation in addition to vascular injury [19,22,23]. Additionally, MMP-2 and -9 are up-regulated following injury, further enhancing the release of TGF- β from the pericellular ECM [24,25].

TGF- β Receptors

Biochemical and genetic studies have established a bi-dimeric receptor system that conveys the TGF- β signal from the cell membrane to intracellular targets. The receptor complex consists of two pairs of transmembrane serine-threonine kinase receptors termed type I and type II receptors. Seven type I, five type II, and two type III receptors have been identified in the human genome that are paired in different combinations for the various ligands of the TGF- β superfamily. The receptor pairs specific for the three TGF- β isoforms are TGF- β receptor I (T β RI also known as activin receptor-like kinase 5 [ALK-5]), TGF- β receptor II (T β RII) [26] and TGF- β receptors III (T β RIII) termed betaglycan and endoglin [21]. TGF- β 1 and TGF- β 3 bind to T β RII whereas TGF- β 2 has a higher affinity to betaglycan, which can then interact with T β RII and T β RI [27]. Interestingly, although BMPs typically signal through activin receptor-like kinase 1 (ALK-1), it has been demonstrated in vascular endothelial cells that TGF- β may also signal through ALK-1 [28]. Moreover, it has been demonstrated that ALK-1 is located primarily in the endothelium whereas ALK-5 is localized to the medial and adventitial layers. Additionally, ALK-1-null mice demonstrate dilated arterial lumens while ALK-5-null mice demonstrate abnormalities in the medial layer and normal arterial lumens [29]. This work suggests functional differences in different TGF- β receptors.

The basic signal flow from the TGF- β ligands through their receptors to their intracellular targets is depicted in Figure 1. Classically, binding of TGF- β to constitutively phosphorylated T β RII causes the complex to bind to and phosphorylate T β RI on specific serine/threonine residues located in the intracellular region adjacent to the cell membrane. T β RI becomes activated and subsequently recruits Smad signaling protein [1,30,31]. T β RI and T β RII can be phosphorylated on tyrosine in addition to serine/threonine, and this can further enhance the non-Smad-dependent Erk and p38 MAP kinase pathways in response to TGF- β [30,32,33].

Receptor degradation is essential to regulating the actions of TGF- β . Through a mechanism involving Smad7, T β RI binds ubiquitin E3 ligase, and undergoes ubiquitination and subsequent degradation [30,34]. WWP1/Tiul1 and NEDD4-2, two additional HECT-type E3 ligases, as well as Smurf1 and Smurf2, two closely related HECT-type E3 ligases, have also been implicated in T β RI degradation *via* a similar mechanism [30,34]. However, the physiological relevance of Smurf1 is unknown as deficiency of Smurf1 has not been found to affect the availability of TGF- β receptor [30,35]. Receptor up-regulation occurs through the covalent binding of SUMO polypeptide (sumoylation) to T β RI which augments the receptor interaction with the Smad signaling proteins [30,36]. Lack of sumoylation has been found to decrease Smad3 activation, and defects in this process have been reported in some breast cancer metastasis [30,36,37].

Smad Signaling Pathway

The Smad signaling pathway is the primary signaling pathway for TGF- β for most gene responses. The Smad signal transducers are cytoplasmic proteins consisting of receptor-regulated Smads (R-Smads 1, 2, 3, 5, and 8), common partner Smads (Co-Smads 4 and 4 β), and inhibitory Smads (I-Smads 6 and 7) that mediate transcription of target genes [1,6,19,38]. TGF- β , as well as activins, nodals, and some GDFs, have classically been thought to signal through Smad2 and 3, whereas BMPs, AMH, and some GDFs signal through Smad1, 5, and 8. Recently, however, TGF- β has been reported to signal through Smad1 and 5 as well [30].

Typically, once TGF- β binds T β RII and subsequently T β RI, Smads2 and 3 are captured and undergo phosphorylation by T β RI at their two serine residues. Phosphorylated Smad2 and 3 then bind Smad4, and the complex is translocated to the nucleus. This then binds to DNA at specific Smad-binding elements and directly recruits transcriptional coactivators or corepressors to target promoters to specific genes [19,39].

Structurally, Smad2 and Smad3 are very similar. Most of the *in vitro* TGF- β responses can be mediated by either Smad. However, animal models have demonstrated the distinct biological functions of these two Smad proteins. Targeted deletions of Smad2 in mice result in early embryonic death, whereas Smad3 knockout mice are viable but die prematurely due to defects in immune function [40,41].

Inhibitor Smads (I-Smads 6 and 7) antagonize TGF- β signaling by competitively inhibiting R-Smad activation or by mediating TGF- β receptor degradation. Smad6 inhibits BMP signaling by blocking binding of R-Smads to common Smad4 [30,42,43] whereas Smad7 inhibits both BMP and TGF- β signaling [30,42,44–47]. Smad7 and T β RI interacts with serine-threonine protein phosphatase 1 (PP1) holoenzyme [44], HECT-type E3 ubiquitin ligase [45], FKBP12 [46], and the salt-inducible kinases (SIK) [47] to promote degradation of TGF- β receptors [30]. Moreover, the inhibitor Smads may also have a role in limiting the intensity and duration of signaling by providing a negative feedback loop and mediating cross-talk with other signaling pathways [1].

Smad Interacting Proteins

Given the complexity and importance of the TGF- β ligands, the basic Smad signaling pathway is surprisingly simple. How can such a simple system mediate a variety of TGF- β responses? The answer lies partially in Smad interacting proteins which regulate an incoming Smad signal at every branching point within the TGF- β pathway; Smad anchor for receptor activation (SARA) is one such protein. SARA stabilizes receptor binding of Smad2 or 3 and enhances clathrin-mediated endocytosis of the complex [30,48,49]. Axin and Dab2 may also act in a similar manner. Axin phosphorylates and stabilizes binding of Smad3 and T β RI. Dab2 binds both T β RI and T β RII, and may play a role in endocytosis of the activated complex to promote TGF- β 's effects [30,50,51]. Additionally, the aforementioned TGF- β type III receptor, betaglycan, is known to increase TGF- β 's affinity for signaling receptors [52].

In addition to inhibitory Smads (I-Smads 6 and 7), a number of proteins have been found to inhibit TGF- β 's interaction with its receptors [52]. For example, FKBP12 and BAMBI prevent type I receptor activation [52–54], which provides fine tuning of this important signaling pathway.

Specific DNA-binding cofactors of the activated Smad complex may represent a group of Smad interacting proteins at the target gene level. These various co-activators or co-repressors mediate recruitment of other transcription factors to Smad target genes, thereby

influencing the nature of TGF- β 's effect [52]. FAST-1, for example, interacts with Smad2/Smad4 or Smad3/Smad4 complexes in the Activin pathway to tightly bind specific DNA sequences [52,55]. CBP and p300, examples of coactivators, alter nucleosome structure to ultimately increase transcription of target genes [52,56–59]. The availabilities of these co-activators or co-repressors ultimately determine which gene or genes will become activated.

Non-Smad Signaling Pathways

In addition to the Smad pathway, TGF- β has been found to signal through Smad-independent but cell type-dependent pathways. The JNK/p38, Erk/MAPK, Rho-like GTPase, and PI3/Akt pathways have been implicated in the epithelial to mesenchymal transition in addition to a number of other gene responses. These non-Smad pathways are believed to reinforce, attenuate or modulate downstream cellular responses possibly accounting for the varying effects of TGF- β [60]. Moreover, there is evidence to suggest that these non-Smad pathways may have significant interactions with the Smad pathway to further explain TGF- β 's diverse functions [39,61]. They may work by directly transforming Smad function or their own function is adapted by Smad signaling [61]. For example, Ras-activated Erk1 and Erk2 protein kinases target linker regions of Smad1, Smad2 and Smad3 to promote nuclear accumulation and therefore inhibit Smad-dependent responses [39,62]. Additionally, it has been found that Smads may be activated by JNK [39,63,64] and activated Smad complexes may interact with Jun complexes [39,65,66].

The complexity of the TGF- β pathway cannot be overstated, as it is reflected at all levels of the signal flow starting from the three TGF- β isoforms to the interactions of Smad proteins with their target genes. The system is further complicated by the existence of multiple regulatory mechanisms at various signaling steps including the Smad proteins. At this time, we have only begun to appreciate the various interactions between Smad and more classical signaling proteins such as MAP kinases, which influence the function or stability of a Smad protein through phosphorylation or other types of modifications. There is no doubt that a more complete understanding of the TGF- β signaling pathway will better explain the often conflicting functions of TGF- β in intimal hyperplasia and arterial remodeling.

SMAD SIGNALING IN VASCULAR INJURY

Smad is Up-regulated at Sites of Arterial Injury

Multiple *in vivo* models have demonstrated that the Smad proteins are up-regulated at sites of arterial injury [67–70]. Sluijter *et al.* demonstrated in rabbit femoral and iliac arteries that activated Smad1, 2, 3, and 5 are up-regulated following balloon injury [67]. LeClair and colleagues utilized the mouse carotid ligation model and found an increased expression of activated Smad2 and 3. Prior to injury, Smad expression was located primarily in endothelial cells and adventitial fibroblasts whereas following injury, Smad expression was up-regulated in adventitial fibroblasts in addition to neointimal SMCs [68]. Using a rabbit abdominal aortic balloon angioplasty model, Zeng *et al.* showed an increased expression of activated Smad2 [69]. The rat carotid injury studies performed in our own laboratory demonstrated a robust up-regulation of Smad3 but not Smad2 in both medial and neointimal cells [71]. In a parallel study, we also found higher Smad3 expression in human restenotic lesions compared with primary atherosclerotic plaques [72]. Since Smad2 and Smad3 are highly homologous but have distinct patterns of gene activation [73], further studies in both human restenosis and animal injury models are necessary to sort out the precise role of these two important TGF- β signaling proteins in regulation of intimal hyperplasia and arterial remodeling.

Overexpression of Smad Increases Intimal Thickening

Following the observation that endogenous Smad3 is up-regulated in injured arteries, we went on to examine the role of Smad3 signaling using the rat carotid model. Adenovirus overexpressing Smad3 was infused into the rat balloon-catheter injured carotid arteries and was found to cause a significant increase in the intima to media ratio compared with arteries treated with empty viral vector [70]. Immunohistochemical analysis revealed an increase in PCNA-positive, or proliferating cells, in the media and neointima of AdSmad3-treated arteries. We postulate that Smad3 may participate in regulation of the phenotypic change of SMCs observed in injured vessels. In support of this hypothesis, we found the majority of cells expressing high levels of Smad3 also bared the proliferation marker [72]. Similar observations were also made in human restenotic lesions [72]. This potential novel function of Smad3 can also be replicated in cultured SMCs. By overexpressing Smad3, we transformed the TGF- β response in vascular SMCs from growth arrest to proliferation [70].

Despite enhanced intimal hyperplasia, AdSmad3-treated arteries showed a larger lumen than the control arteries, indicating a potential role of Smad3 in adaptive remodeling [71]. Evidence obtained from both *in vitro* and *in vivo* experiments suggest a cell-cell communication between medial smooth muscle cells and adventitial fibroblasts that is mediated by connective tissue growth factor (CTGF). Although well-established as a Smad3 target gene [74], not much has been learned about this potent pro-fibrotic factor during vascular injury. Contrarily, studies on TGF- β and dermal wound healing may provide additional insight into the mechanisms by which TGF- β stimulates fibrosis. Lopes *et al.* has demonstrated *in vitro* that TGF- β -induced CTGF expression occurs at least in part in a Smad-independent pathway [75] as opposed to the classic Smad pathway [74]. The authors treated cultured human keloid fibroblasts with TGF- β and AZX100, a known inhibitor of TGF- β -induced CTGF expression, and evaluated the activation of Smad3. The investigators found that AZX100 did not affect Smad3 activation thereby suggesting a Smad-independent mechanism by which collagen I expression is inhibited [75].

We found that adventitial application of exogenous CTGF led to similar arterial remodeling as observed in AdSmad3-treated carotid arteries [71] as well as in TGF- β 3-treated coronary arteries [10]. Whether Smad3 and its target gene CTGF are responsible for the TGF- β 3-induced outward remodeling demonstrated in the Kingston study remains to be investigated. However, both our laboratory and the Kingston group found increased collagen accumulation associated with outward remodeling in Smad3-, CTGF-, or TGF- β 3-treated vessels [10,71]. These findings, although inconsistent with the pro-fibrotic functions of TGF- β 3, Smad3, and CTGF, challenged an earlier view that constrictive remodeling results from collagen synthesis or vascular fibrosis. Additionally, although Jiang *et al.* found that TGF- β and CTGF are up-regulated in a rabbit vein graft model and that this was associated with myofibroblast transformation and cell migration, they argue that this leads to attenuation of outward modeling [76].

Although TGF- β is well-established as a pro-fibrotic factor, the downstream signaling proteins that mediate TGF- β -induced expression of ECM proteins are not always obvious. Using a Smad4-deficient cell line, MDA-MB-468, Itoh *et al.* demonstrated that fibronectin expression is mediated by non-Smad-dependent pathways [77]. However, in cultured vascular SMCs, Smad7 blocks TGF- β -induced expression of fibronectin, collagen and CTGF [78]. We have also demonstrated the Smad-dependency of fibronectin in cultured smooth muscle cells [79].

Blockade of Smad Attenuates Intimal Hyperplasia

A corollary to our studies that implicate Smad3 in restenosis is the use of its inhibitor to decrease this hyperplastic response. We have performed balloon-catheter injury in rat carotid arteries followed by adenovirus overexpressing Smad7, the known inhibitor of the TGF- β /Smad3 pathway. AdSmad7 attenuates neointimal formation that is associated with reduction in medial and intimal cell proliferation [70]. Similar reduction in intimal hyperplasia was also accomplished by Mallawaarachichi *et al.* who applied adenovirus expressing Smad7 perivascularly to rat balloon-catheter injured carotid arteries. The authors found, however, that Smad7 application was associated with decreased Smad2 phosphorylation leading to decreased adventitial cell migration to neointima and collagen deposition [80]. Although the structures of Smad2 and Smad3 are similar, their functions may differ as evidenced by early embryonic death of Smad2 knockout-mice [40,41]. However, our knowledge of the specificities of these two Smads in their functions is limited, which prevents a better understanding of their roles in vascular diseases.

Smad Knock-Out Mice Demonstrate Increased Intimal Hyperplasia

The use of Smad7 to block Smad3 effects differs from studies utilizing Smad3 knock-out mice as Smad7 would inhibit both Smad2 and 3 whereas Smad3 knock-out mice, on the other hand, are a selective and complete loss of Smad3. Moreover, Smad3 knock-out mice demonstrate a global and embryonic loss of Smad3 which may or may not affect arterial injury. Nevertheless, Kobayashi *et al.* injured the femoral arteries of Smad3-null mice by photochemically-induced thrombosis and found that they exhibited exaggerated neointimal hyperplasia compared with wild-type mice, and this was attributed to increased cell proliferative activity of Smad3-null SMCs. However, the authors performed a bone marrow transplant of Smad3-null bone marrow to wild-type mice and performed photochemical-induced thrombosis on the host mice. These mice did not demonstrate an increase in neointimal hyperplasia. The authors suggest that this is due to resident vascular cells being the primary cells responsible for the increase in cell proliferation. Contrary to our findings that Smad3 is a critical mediator of restenosis, these authors propose that endogenous Smad3 plays a protective role following vascular injury [81]. The potential effect of Smad2 gene deficiency on vascular injury response is unknown due the embryonic lethality of Smad2 knockout.

Potential Role of TGF- β /Smad Signaling in Progenitor Recruitment and Differentiation

Recent data has emerged implicating bone marrow derived progenitor cells (BMPCs) into sites of vascular injury in addition to the VSMCs located within the vessel wall [82,83]. Although the exact mechanisms by which BMPCs are recruited to sites of injury and differentiate into VSMCs have yet to be elucidated, many investigators have confirmed that BMPCs contribute to the neointima, at least in mouse models, to varying degrees [83].

In vitro data suggest that TGF- β possesses the ability to stimulate the secretion of chemokines involved in BMPC recruitment as well as differentiation. Chen *et al.* demonstrated in neural crest stem cells that TGF- β , *via* Smad3 signaling, promotes differentiation into smooth muscle cells (SMCs) [84]. More recently, Imamura *et al.* showed that TGF- β induces the differentiation of bone marrow-derived endothelial progenitor cells (EPC) toward SMC lineage [85]. Although the involvement of TGF- β in the recruitment of progenitor cells to injured arteries remains to be tested *in vivo*, our recent *in vitro* studies demonstrate that TGF- β stimulated VSMCs, also *via* Smad3, to produce a soluble factor(s) that recruit(s) bone marrow-derived progenitor cells [60]. Through a gene array analysis, we have identified monocyte chemoattractant protein-1 (MCP-1), a chemokine well-known to recruit monocytes to sites of inflammation and injury, as a potential chemoattractant for progenitor cells. Blocking MCP-1 production in SMCs with an siRNA, we were able to

attenuate the ability of Smad3-expressing SMCs to recruit progenitor cells [22]. However, we detected few inflammatory cells in injured rat carotid arteries following Smad3 infection despite up-regulation of MCP-1 [60,83]. The effect of Smad3 on MCP-1 expression is likely cell-type specific. For example, Feinberg and colleagues showed that TGF- β /Smad3 is a negative regulator of MCP-1 expression in macrophages. The authors demonstrated that cardiac allografts in Smad3-deficient mice developed accelerated intimal hyperplasia with increased infiltration of adventitial macrophages expressing MCP-1 [86].

THERAPEUTIC TARGETING OF TGF- β SIGNALING PATHWAYS: OPPORTUNITIES AND CHALLENGES

As the role of TGF- β and its signaling components in cardiovascular diseases is increasingly recognized, there is a growing interest in TGF- β as a therapeutic target. Several classes of inhibitors have been developed to antagonize the TGF- β pathway; many of which target ligand-receptor interactions. Examples of such inhibitors include anti-TGF- β antibodies, anti-receptor antibodies, TGF- β -trapping receptor ectodomain proteins, and small molecule inhibitors of TGF- β receptors. With the development of RNA interfering technology and nanotechnology, molecular agents targeting various Smad proteins could provide additional inhibitory strategies.

Targeting TGF- β in restenotic disease is not only supported by a vast body of experimental data but also by the fact that some of the TGF- β inhibitors have entered clinical trials for efficacy against cancers and fibrosis. However, we need to be very cautious in applying an anti-TGF- β strategy to restenotic patients as these patients may have underlying pathology such as atherosclerosis, diabetes or even occult malignancy. A major concern is the potential effect of an anti-TGF- β therapy on plaque instability and thrombosis. Once again, TGF- β 's role here is complicated. In humans, plasma TGF- β concentration has been found to be negatively correlated with atherosclerosis [87–90]. Animal studies utilizing systemic suppression of TGF- β activity with neutralizing antibodies to TGF- β 1, - β 2 and - β 3 or with a soluble TGF- β -receptor II protein have been found to accelerate the development of atherosclerosis and induce an unstable plaque phenotype [91,92].

Undoubtedly, many challenges exist in the pathway of translating anti-TGF- β strategies to therapies for restenosis, and its primary limitation in success in clinical trials may be due to an incomplete understanding of TGF- β biology. Since TGF- β is a potent growth repressor for many types of cells, whether an anti-TGF- β therapy could lead to unwanted tumor growth is a sobering concern. Additionally, TGF- β function is context-dependent with differences in outcome related to the availability of its co-activators and co-repressors, ligand concentration, composition of its isoforms, cell type and concentration, and the various crosstalks associated with its primary signaling pathway Smad. In this regard, targeting downstream signaling proteins such as Smad, rather than ligand or receptor, might offer a selective attenuation of an unwanted TGF- β function and therefore a safer therapy to treat intimal hyperplasia. However, our understanding of the specificities of the Smads is very limited in vascular disease due to the lack of reliable approaches to selectively assess their function. This will present as a challenge when developing anti-Smad therapies.

It is important to note that a 'TGF- β paradox' exists. In cancer cells, TGF- β functions as a tumor suppressor initially and switches to a tumor promoter later [93]. A similar paradox exists in TGF- β 's role in inflammation [94]. Another important aspect is perhaps the temporal relationship between TGF- β up-regulation and other pathologic events. In restenosis, inflammation occurs initially followed by smooth muscle cell proliferation; TGF- β expression is followed by Smad expression. Therefore targeting TGF- β at specific time points during the disease process may be an option worth considering.

Vessel-specific drug delivery system such as catheter- or stent-based drug delivery devices may avoid unnecessary disruption of TGF- β signaling in other organ systems. As described in the various animal models employed by the authors cited, there are a number of techniques to deliver anti-TGF- β therapies with specific advantages. Intraluminal delivery is frequently employed and may be easier to translate to human use as in catheter- or stent-based delivery. This method targets primarily smooth muscle cells. The use of periadventitial application of therapies targets mainly periadventitial cells and may be used in conjunction with open surgery. Systemic therapies, while the easiest to use, are non-specific. However, this limitation could be overcome with the use of tissue-specific targeting through the so-called smart drug design, an approach requiring identification of molecular markers that are differentially up-regulated in restenotic vessels.

Therapeutic targeting of the TGF- β pathway in injured vessels is based on the rationale that TGF- β promotes intimal hyperplasia. However, some components of the TGF- β pathway may favor outward or adaptive remodeling, as shown by Kingston's TGF- β 3 study [10] and by our own Smad3 study [70]. Additionally, the TGF- β /Smad signal may lead to different biological outcomes in different cell types as shown by Feinberg's MCP-1 study in macrophages [86] and ours in SMCs [60]. Therefore, a better understanding of various TGF- β ligands and their signaling mechanisms following vascular injury is crucial. Finally, targeting TGF- β might cause chronic inflammatory and autoimmune reactions, which could potentially worsen atherosclerosis or diabetes, conditions that are frequently associated with restenotic patients.

As we progress toward individualized medicine, studies designed to delineate the multifunctional effects and mechanisms of TGF- β in different restenotic lesions (for example: coronary versus peripheral, carotid versus femoral, and arterial versus venous) are essential. Equally important is the evaluation of TGF- β function in patients associated with different risk factors such as diabetes, smoking, and aging to determine when and how anti-TGF- β therapy might be feasible. For example, TGF- β levels have been found to be elevated in patients with diabetes mellitus type I [95] and have been correlated with an increased risk of developing diabetes mellitus type II [96]. Moreover, it has been implicated in the pathogenesis of diabetes nephropathy [97,98], and cigarette smoke may produce an additive effect in its development [99]. On the contrary, TGF- β has been associated with longevity [100] and healthy individuals compared with Alzheimer's patients [101]. The idea is that restenotic lesions developed in different vascular beds or in different individual patients may have different underlying pathophysiology. Therefore, we need to develop diagnostic tools that allow the analysis of TGF- β signaling and measurable downstream effectors of TGF- β 's functions in individual patients, which will ultimately enable us to identify and select patients who would possibly derive the most benefit from an anti-TGF- β therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1.

Basic TGF- β /SMAD signaling: A TGF- β ligand binds to and forms a receptor complex with TGF- β receptor type II and type I. Receptor Smad (R-Smad) is then recruited along with Smad anchor for receptor activation (SARA), which stabilizes receptor and R-Smad binding. The TGF- β receptors activate R-Smad through phosphorylation at the COOH-terminal and combines with Co-Smad to enter the nucleus. The complex then binds to specific DNA sequences of target genes. In collaboration with other transcriptional modulators, TGF- β /receptor interaction also leads multiple non-Smad pathways which reinforce, attenuate or modulate downstream cellular responses of TGF- β .

TABLE 1

The History of TGF- β and Restenosis

TGF-β is up-regulated following injury				
Experimental model	Determination of TGF-β expression	Time	Proposed mechanism	Publication
Rat carotid artery balloon-catheter injury	IHC	10 wk	ECM synthesis	Madri et al. 1989 [2]
Rat carotid artery balloon-catheter injury	IHC and RNA isolation and blot hybridization	2 wk	ECM synthesis and SMC proliferation	Majesky et al. 1991 [3]
Human primary atherosclerotic, nonatherosclerotic and restenotic lesions	IHC and ISH		SMC proliferation	Nikol et al. 1992 [4]
Human coronary arteries with stent restenosis	IHC		SMC proliferation	Yutani et al. 1999 [5]
Porcine coronary angioplasty injury	IHC and modified PAI-1/L assay	4 wk	Active TGF-β up-regulation occurs after injury	Chamberlain et al. 2001 [6]
Up-regulation of TGF-β increases intimal thickening				
Experimental model	Major Findings		Time	Publication
TGF-β gene transfer to uninjured porcine iliofemoral arteries	TGF-β-treated arteries demonstrate a 10-fold increase in I:M ratio when compared to control-treated arteries		3 wk	Nabel et al. 1993 [7]
Systemic TGF-β to rabbits that have undergone carotid artery balloon-catheter injury	TGF-β-treated animals demonstrate a nearly 2-fold increase in I:M ratio when compared to aspirin-treated animals		4 wk	Kanzaki et al. 1995 [8]
TGF-β gene transfer to vein graft into rat femoral artery	TGF-β-treated vein grafts demonstrate a 16-fold increase in collagen content when compared to control-treated vein grafts		12 wk	Wolff et al. 2006 [9]
TGF-β gene transfer to porcine angioplastied coronary arteries	TGF-β1-treated arteries demonstrate a 3-fold decrease in neointimal area when compared to TGF-β3-treated arteries		3 d	Kingston et al. 2003 [10]
Blockade of TGF-β attenuates intimal thickening				
Systemic TGF-β antibody to rats that have undergone carotid artery balloon-catheter injury	Anti-TGF-β antibody-treated arteries demonstrate a 1.5-fold decrease in I:M ratio when compared to control antibody-treated arteries		19 d	Wolf et al. 1994 [11]
Ribozyme oligonucleotide to TGF-β to rat balloon-catheter injured arteries	Ribozyme-treated arteries demonstrate a 4-fold decrease in neointimal area when compared to control injured arteries		2 wk	Yamamoto et al. 2000 [12]
Systemic soluble TGF-β receptor II to rats that have undergone carotid artery balloon-catheter injury	TGF-βRII-treated arteries demonstrate a 1.25–2.78-fold decrease in I:M ratio when compared to control injured arteries in a dose-dependent manner		2 wk	Smith et al. 1999 [13]
TGF-β receptor II gene transfer to porcine angioplastied coronary arteries	TGF-βRII-treated arteries demonstrate a 1.3-fold increase in luminal area when compared to control virus-treated arteries		4 wk	Kingston et al. 2001 [14]

IHC = immunohistochemistry; ECM = extracellular matrix; SMC = smooth muscle cell; ISH = *in situ* hybridization; PAI-1/L = plasminogen activator-inhibitor/luciferase; I:M = intima to media; FB = fibroblasts.