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Further Evidence Supporting a Protective Role of Transforming Growth Factor-β in Aortic Aneurysm and Dissection

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Aortic disease arises from abnormalities in size and/or structure of the vessel wall. A (fusiform) aneurysm is a localized dilatation of the aorta, usually defined as greater than 150% of the normal diameter for a given segment. Aortic dissection is bleeding into the media layer, often with propagation of a false lumen. Both diseases can occur independently, although dilated aortas are at increased risk of dissection, dissected aortas have increased expansion rates, and either process can result in vessel rupture. Aneurysms and dissections are broadly classified as affecting the thoracic (supradiaphragmatic) or abdominal (infradiaphragmatic) aorta. Thoracic aortic disease is characterized by medial degeneration, whereas pathology of the abdominal aorta includes substantial inflammatory infiltrates, marked loss of smooth muscle cells (SMCs), and frequent luminal thrombus. Additionally, thoracic but not abdominal aneurysm and dissection is associated with numerous genetic mutations, including genes coding for fibrillin-1 causing Marfan syndrome or components of the transforming growth factor-β (TGFβ) signaling pathway causing Loeys-Dietz syndrome, such as TGFβ receptors, TGFβ ligands, and SMAD transcription factors.

Mouse models have recapitulated many pathological aspects of aortic aneurysm and dissection and are informative in testing mechanisms of disease and potential therapeutics. A popular model is the infusion of angiotensin II (AngII) to hypercholesterolemic mice, first described by the Daugherty group. Dissection of the suprarenal abdominal aorta occurs within 4–10 days in the majority of animals and aneurysms restricted to this region progressively develop from vascular remodeling over several weeks [1]. Normocholesterolemic mice infused with AngII have less abdominal but similar thoracic aortic disease manifesting as infrequent dissection and rupture with modest dilatation of the ascending aorta by 7 days [2,3]. Although a convenient experimental model, there is limited evidence for AngII hyperactivity in clinical disease. Hence, AngII inhibitors are not prescribed for patients with aortic aneurysm and dissection except as antihypertensives [4]. Moreover, AngII-mediated aortic disease is driven by severe inflammation and marked

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thickening of the vessel wall that differs from typical thoracic aortic disease in patients. Transgenic engineering of mice for mutant *Fbn1* and genes encoding TGFβ signaling molecules provide additional models of thoracic aortic disease more firmly based on clinical pathogenesis. Initial studies in AngII-infused or *Fbn1*$^{C1041G/+}$ mice demonstrated that inhibition of TGFβ signaling by neutralizing antibodies or pharmacological agents modestly inhibited thoracic aortic aneurysm formation [5–7]. However, several subsequent studies have shown that TGFβ neutralization markedly increased aortic aneurysm size and rupture in AngII-infused or *Fbn1*$^{mgR/mgR}$ mice [8–10] and that conditional deletion of *Tgfbr2* in smooth muscle cells greatly induced spontaneous aortic aneurysm and dissection and aggravated the aortopathy of *Fbn1*$^{C1041G/+}$ mice [11–13]. Thus, there is conflicting evidence for pathogenic versus protective roles for TGFβ in aortic disease. The field is further complicated by the paradoxical activation of TGFβ signaling driving aortic disease in certain mouse strains with deficiency or loss-of-function mutations of *Tgfb2*, *Tgfbr1*, *Tgfbr2*, and *Smad3* in which attenuated TGFβ signaling is predicted [14–17].

Addressing this controversial subject in the current issue of *ATVB*, Angelov et al. report that systemic neutralization of TGFβ worsens abdominal but not thoracic aortic disease, whereas conditional deletion of TGFβ signaling in SMCs exacerbates thoracic but not abdominal aortic disease [18]. The findings of this new study by the Dichek group compared to previous studies are summarized in Table 1. Although it has previously been shown that TGFβ protects the abdominal aorta from AngII-mediated disease through effects on cell types other than SMCs [8] and that TGFβ signaling in SMCs protects the thoracic aorta from spontaneous or mutant *Fbn1*-mediated disease [11–13], the advance of this new study is that the effects of systemic and conditional inhibition of TGFβ signaling in both the thoracic and abdominal aorta are compared in the same murine model of AngII-mediated aortic disease. However, key differences with previous studies are highlighted by the authors in which they consider technical factors as explanations. For example, Wang et al. and Chen et al. found that administration of neutralizing TGFβ antibody increased the size and rupture of thoracic aortas (in addition to that of abdominal aortas) in AngII-infused mice [8,9]. The Dichek group also previously reported that SMC-specific *Tgfbr2* deletion induced infrequent abdominal aortic disease (as well as frequent thoracic aortic disease) without AngII infusion [12]. These prior findings contradict the novel conclusion reached by Angelov et al. that SMC-extrinsic TGFβ signaling causes abdominal aortic disease while SMC-intrinsic TGFβ signaling causes thoracic aortic disease. Therefore, the techniques used to (i) induce, (ii) assess, and (iii) modulate aortic disease in mice merit further consideration in explaining differences between studies and to promote a standard approach in this disputed field.

(i) Disease Models and Host Factors

AngII infusion induces a vigorous inflammatory infiltrate that drives vascular remodeling predominantly of the abdominal aorta [1,2], whereas aortopathy attributable to *Fbn1* mutations or SMC-specific *Tgfbr2* deletion has less inflammation and largely affects the thoracic aorta [5,6,11–13]. Germline deletion of *Smad3* leads to significant leukocytic activation and a more severe thoracic aorta phenotype including rupture [15], likely due to dual SMC-intrinsic and -extrinsic effects. Selection of a particular model will thus favor certain disease mechanisms in limited aortic segments. In other words, it is not surprising

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that systemic neutralization of TGFβ that readily accesses circulating leukocytes worsens inflammation and AngII-mediated abdominal aortic disease. Similarly, selective disruption of TGFβ signaling in SMCs may be expected to preferentially affect thoracic aortic pathology independent of immune responses or AngII effects on the abdominal aorta. Furthermore, classification of aortic disease as either thoracic or abdominal is overly broad as differences in pathology within the aortic root, ascending aorta, aortic arch, descending thoracic aorta, suprarenal abdominal aorta, and infrarenal abdominal aorta have been described [2,3,11,16]. Regional differences in hemodynamic loads, embryological origin of SMCs, matrix composition, receptor distribution, etc. may contribute to disease localization. Although AngII infusion causes severe disease of the suprarenal but not infrarenal abdominal aorta in mice, the experimental findings are often extrapolated to abdominal aortic aneurysms in patients with a reverse pattern of disease. Distinguishing only between thoracic versus abdominal locales also fails to account for certain clinical similarities between descending thoracic and abdominal aortic aneurysms as opposed to proximal aortic aneurysms. Disease severity at all aortic regions is determined by the age, sex, and genetic background of the mice as well as the dose and duration of AngII administration [19]. Angelov et al. aptly consider genetic drift of colonies and the gut microbiome as additional factors that may contribute to variable outcomes.

(ii) Control Groups and Diagnostic Methods

Since aortic aneurysms are defined by comparison to normal vessels, untreated controls are necessary. In contrast, any degree of medial bleeding is considered abnormal. It is unwise to rest on historical controls for normal vessel diameters, even in inbred strains, given the variations due to host factors. The complexity of background strain variations when breeding compound mutant mice and possible differences in the intestinal microbiome mandate the use of littermate controls as Angelov et al. employed. However, they elected not to use untreated control groups and compared the effects of inhibiting TGFβ signaling only in AngII-infused mice. This approach does not allow for the diagnosis of aortic aneurysm as a function of normal vessel diameter; instead conclusions are limited to whether altered TGFβ signaling modulates AngII-driven aortic disease. As implemented by Angelov et al., observer bias needs to be minimized by blinded assessments, since the experimental design (i.e., strategies to inhibit or exacerbate disease) also influences the severity of AngII-mediated aortic disease [19]. Imaging studies provide physiological measurements of aortic size in vivo, but ultrasound is not applicable to all regions of the aorta due to suboptimal windows from air-filled organs and CT or MRI scans are not practical for large numbers of animals. Moreover, imaging studies may underestimate the incidence of modest medial bleeding. Direct inspection is valuable in assessing the unpressurized size of all regions of the aorta and diagnosing aortic dissection, but cannot be performed serially or in live animals, may not differentiate between medial versus adventitial bleeding, and may miss minor dissection or medial hemorrhage that resolves within several weeks. Although subject to fixation artifacts, histology can further define mechanisms of aortic size changes by assessment of individual vessel wall compartments. False aneurysms, or bleeding contained by the adventitia, can contribute to enlargement of the aorta as noted by Angelov et al. Special stains may confirm minor medial bleeding by markers for red blood cells or reveal

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evidence of resolving medial hemorrhage by ferric iron deposition [11,12]. Ideally, all three modalities of in vivo, in situ, and ex vivo measurements should be performed at both early time points (around 7 days) to assess aortic dissection and late time points (around 4 weeks) to assess aortic aneurysm. The more limited assessment by Angelov et al. may have missed minor or resolving aortic disease.

(iii) Inhibition of TGFβ Signaling

Genetic and serological inhibition of TGFβ signaling is not equivalent in selectivity or efficacy. Conditional disruption of *Tgfbr2* requires robust and specific construct expression. The *Acta2* promoter is less specific for SMCs than that of *Myh11*, e.g., expression by myofibroblasts [20], and effects on cell types other than SMCs cannot be excluded. This is important as AngII-mediated aortic disease involves a number of cell types directly or indirectly, including fibroblasts, endothelial cells, lymphocytes, and macrophages [8, 21,22]. The disadvantage of the available *Myh11-CreER* strain is that only male mice can be studied due to construct insertion in the Y chromosome unlike autosome expression of *Acta2-CreER*. Because aberrant TGFβ signaling may occur following either *Tgfbr1* or *Tgfbr2* deletion [23,24], it is warranted to exclude this possibility by assessment of TGFβ signaling, concomitant TGFβ neutralization, or use of compound *Tgfbr1/2* deficient animals. Assessing the expression of TGFβ receptors may be problematic due to their relatively low abundance and the presence of cells other than SMCs within the aortic wall, particularly after marked vascular remodeling as encountered by Angelov et al. The use of reporter constructs can be invaluable in confirming successful genetic recombination in each relevant aortic segment of every experimental subject [11]. Greater than 50% inhibition of TGFβ signaling is required to disrupt SMC homeostasis, as aortopathy does not result from *Tgfbr2* haploinsufficiency. Superimposing systemic TGFβ neutralization on SMC-specific *Tgfbr2* deletion may indicate SMC-independent effects [11]. The efficacy of neutralizing antibodies is clone and dose dependent [8]. Rare pan-reactive antibodies have differing affinities for individual TGFβ isoforms. Inhibition of TGFβ signaling by neutralizing antibodies may also vary in different tissue compartments. The aortic wall with an intact endothelium restricts the transport of macromolecules such as immunoglobulins into the media [25]. Several studies, including that of Angelov et al., assess neutralizing antibody efficacy by measuring circulating TGFβ. Serum levels are problematic as plentiful TGFβ stored in platelet granules is released during clot formation. This pool of intracellular TGFβ may be less accessible to circulating antibody and platelet-poor plasma levels are preferred. TGFβ epitopes for specific antibody binding can be obscured when the cytokine is bound to its latency-associated peptide. Neutralizing antibodies such as 2G7 bind the active form of TGFβ [26] and quantification of total (active and inactive) TGFβ following acidification of serum may be misleading due to the vast pool of latent cytokine. Controls are also required to determine if binding of neutralizing antibody to TGFβ influences the detection of TGFβ by antibody-dependent ELISA techniques. This phenomenon of cross-blocking may explain the discrepant neutralization of TGFβ isoforms using well-characterized antibodies as performed by Angelov et al.; receptor binding assays or functional assays of TGFβ signaling and transcriptional/translational responses are preferable. The local activation of latent cytokine further precludes assumptions of TGFβ signaling in extravascular cells from

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determination of circulating levels. Although not pursued by Angelov et al., it is optimal to assess TGFβ activity at the target tissue level. Western blotting has the advantage of distinguishing nonspecific labeling by molecular weight but represents a global assessment of tissue effects. Immunohistochemistry has good spatial discrimination though relevant controls are essential to exclude nonspecific binding. Limitations to assessing phosphorylated forms and/or nuclear translocation of SMAD2/3 are that other TGFβ superfamily members, such as activins, nodal, and (some) growth and differentiation factors, use the same transcription factor intermediaries and that stress-related signaling may promiscuously activate SMAD2 in SMCs of *Fbn1*-null mice independent of TGFβ receptor activity [27]. Since signaling events are rapid, expeditious processing of the aortic tissue is of great importance as artifacts may ensue in response to the withdrawal of hemodynamic forces or the mechanical stimulation of excising the adventitia. Basal TGFβ signaling within vessel wall cells may also vary with age. Pathological changes of the aorta resulting from disruption of TGFβ signaling in SMCs is critically dependent on the postnatal developmental stage of the animal [11] and TGFβ neutralization displays dimorphic effects on mutant *Fbn1*-mediated aortic disease depending on the age of the host and disease onset [10]. Finally, TGFβ has varying effects on different vessel wall cell populations [28] and even on SMCs of different embryological origin [29] suggesting that its signaling effectors are not universal.

Within the confines of the above caveats, Angelov et al. show that TGFβ signaling in SMCs protects against thoracic aortic disease and in cells other than SMCs protects against abdominal aortic disease. To further test this interesting hypothesis, TGFβ signaling should be deleted in additional cell types that contribute to vessel wall homeostasis. Although complete disruption of signaling in specific cell types is mechanistically informative, partial inhibition of signaling in all cells is of therapeutic relevance. This new study finds a consistent benefit for TGFβ activity in aortic disease and contributes to the growing body of evidence against the once promising approach of inhibiting TGFβ signaling in patients with aortic aneurysm or at risk of aortic dissection.

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Table 1

Effects of attenuated TGFβ signaling on aortic disease in mice.

Study	Strain	Agent	TGFβ Inhibition	Thoracic Aorta	Abdominal Aorta
Angelov et al., 2017 [18]	WT	AngII	mAb	↔ size, ↔ dissection	↑ size, ↑ dissection
	Tgfbr2$^{-/-}$	AngII	genetic	↔ size, ↑ dissection	↔ size, ↔ dissection
King et al., 2009 [7]	*Apoe*$^{-/-}$	AngII	pAb	N.A.	↔ size
	Apoe$^{-/-}$.*Cxcl10*$^{-/-}$	AngII	pAb	N.A.	↓ size
Wang et al., 2010 [8]	WT	AngII	pAb or mAb	↑ size/dissection, ↑ rupture	↑ size/dissection, ↑ rupture
	Apoe$^{-/-}$	AngII	pAb or mAb	N.A.	↑ rupture
Chen et al., 2016 [9]	WT	AngII	pAb	↔ size, ↔ rupture	↔ size, ↔ rupture
	WT	AngII	mAb	↑ size, ↑ rupture	↑ size, ↑ rupture
Habashi et al., 2006 [5]	*Fbn1*$^{C1041G/+}$	None	pAb	↓ size	N.A.
	Fbn1$^{C1041G/+}$	None	Losartan	↓ size	N.A.
Holm et al., 2011 [6]	*Fbn1*$^{C1041G/+}$	None	RDEA119	↓ size	N.A.
Cook et al., 2015 [10]	*Fbn1*$^{mgR/mgR}$	None	mAb at P16	↑ size, ↑ rupture	N.A.
	Fbn1$^{mgR/mgR}$	None	mAb at P45	↓ rupture	N.A.
Li et al., 2014 [11]	*Tgfbr2*$^{-/-}$	None	genetic	↑ size, ↑ dissection	rare dissection
	Tgfbr2$^{-/-}$	None	genetic + mAb	↑ rupture	N.A.
	Fbn1$^{C1041G/+}$.*Tgfbr2*$^{-/-}$	None	genetic	↑ size, ↑ dissection	N.A.
Hu et al., 2015 [12]	*Tgfbr2*$^{-/-}$	None	genetic	↑ size, ↑ dissection	↑ size, few dissections
Wei et al., 2017 [13]	*Fbn1*$^{C1041G/+}$.*Tgfbr2*$^{-/-}$	None	genetic	↑ size, ↑ dissection	N.A.

Experimental murine studies investigating the effects of decreased TGFβ signaling on aortic aneurysm and dissection. All mice were on a C57BL/6 background. Aortic disease was induced or modulated by infusion of the vasoconstrictor agent AngII, apolipoprotein E deficiency (*Apoe*$^{-/-}$), *Cxcl10* deficiency (*Cxcl10*$^{-/-}$), *Fbn1* mutations (*C1041G* or *mgR*), or conditional deletion of *Tgfbr2* in SMCs (*Tgfbr2*$^{-/-}$). The *C1041G* mutation in mice is also known as *C1039G* based on a similar mutation causing Marfan syndrome in humans. TGFβ signaling was inhibited by administration of neutralizing polyclonal (pAb) or monoclonal (mAb) antibodies (initiated at P16 vs. P45 of age in one study), the AngII receptor blocker losartan, the ERK1/2 inhibitor RDEA119, or SMC-specific deletion of *Tgfbr2*. Thoracic and abdominal aortic size, dissection, and rupture was documented as increased (↑), decreased (↓), no change (↔), few/rare, or not assessed (N.A.).