EBioMedicine xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Defective Connective Tissue Remodeling in Smad3 Mice Leads to Accelerated Aneurysmal Growth Through Disturbed Downstream TGF-β Signaling

I. van der Pluijm, PhD [a,b,c], N. van Vliet, BSc [b], J.H. von der Thusen, PhD [d], J.L. Robertus, PhD [d], Y. Ridwan, BSc [b], P.M. van Heijningen, BSc [b,c], B.S. van Thiel, MSc [a,b,e], M. Vermeij, BSc [b], S.E. Hoeks, PhD [f], R.M.G.B. Buijs-Offerman, PhD [c], H.J.M. Verhagen, MD, PhD [a], R. Kanaar, PhD [b,g], A.M. Bertoli-Avella, MD, PhD [c,1], J. Essers, PhD [a,b,g,*]

[a] Department of Vascular Surgery, Erasmus University Medical Center, Rotterdam, The Netherlands
[b] Department of Molecular Genetics, Cancer Genomics Netherlands, Erasmus University Medical Center, Rotterdam, The Netherlands
[c] Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands
[d] Department of Pathology, Erasmus University Medical Center, Rotterdam, The Netherlands
[e] Department of Pharmacology, Erasmus University Medical Center, Rotterdam, The Netherlands
[f] Department of Anesthesiology, Erasmus University Medical Center, Rotterdam, The Netherlands
[g] Department of Radiation Oncology, Erasmus University Medical Center, Rotterdam, The Netherlands

ARTICLE INFO

Article history:
Received 6 June 2016
Received in revised form 29 August 2016
Accepted 8 September 2016
Available online xxxx

Keywords:
Aneurysm
Vascular biology
TGF-β signaling pathway
Echocardiography
Mouse model

ABSTRACT

Aneurysm-osteoarthritis syndrome characterized by unpredictable aortic aneurysm formation, is caused by *SMAD3* mutations. SMAD3 is part of the SMAD2/3/4 transcription factor, essential for TGF-β-activated transcription. Although TGF-β-related gene mutations result in aneurysms, the underlying mechanism is unknown. Here, we examined aneurysm formation and progression in *Smad3*$^{-/-}$ animals.

Smad3$^{-/-}$ animals developed aortic aneurysms rapidly, resulting in premature death. Aortic wall immunohistochemistry showed no increase in extracellular matrix and collagen accumulation, nor loss of vascular smooth muscle cells (VSMCs) but instead revealed medial elastin disruption and adventitial inflammation. Remarkably, matrix metalloproteases (MMPs) were not activated in VSMCs, but rather specifically in inflammatory areas. Although *Smad3*$^{-/-}$ aortas showed increased nuclear pSmad2 and pErk, indicating TGF-β receptor activation, downstream TGF-β-activated target genes were not upregulated. Increased pSmad2 and pErk staining in pre-aneurysmal *Smad3*$^{-/-}$ aortas implied that aortic damage and TGF-β receptor-activated signaling precede aortic inflammation. Finally, impaired downstream TGF-β activated transcription resulted in increased *Smad3*$^{-/-}$ VSMC proliferation.

Smad3 deficiency leads to imbalanced activation of downstream genes, no activation of MMPs in VSMCs, and immune responses resulting in rapid aortic wall dilatation and rupture. Our findings uncover new possibilities for treatment of *SMAD3* patients; instead of targeting TGF-β signaling, immune suppression may be more beneficial.

1. Introduction

Aortic aneurysms significantly increase the risk of tearing (dissection) or rupture of the aorta with life-threatening consequences. As for several cardiovascular diseases, male gender, advanced age, and positive family history are among the main risk factors for developing aneurysms (Albornoz et al., 2006; Grubb and Kron, 2011). Multiple genes for thoracic aortic aneurysms have been identified (Gillis et al., 2013), but most of the underlying genetic and molecular interactions are not known. These genes mainly fall into three categories, based on their function; 1) extracellular matrix integrity and structure, 2) involvement in TGF-β signaling, and 3) cytoskeleton maintenance and mobility. Although their functions are quite different, defects in these genes all lead to aneurysm formation.

The extracellular matrix (ECM) is important for the integrity of the aortic wall and mutations in ECM genes such as Fibulin-4 and Fibrillin-1 hence lead to aneurysm formation. Genetic studies in Fibulin-4 and Fibrillin-1 mutant mice implicated dysregulation of the TGF-β pathway as an important hallmark in the pathogenesis of aneurysm formation, both in mice and humans (Neptune et al.,

Abbreviations: AOS, aneurysm-osteoarthritis syndrome; CTGF, connective tissue growth factor; ECM, extracellular matrix; MFS, Marfan's syndrome; MMP, matrix metalloproteases; TGF-β, transforming growth factor β; VSMC, vascular smooth muscle cell; LDS, Loeys-Dietz syndrome; SMAD, SMA/MAD homologous.

* Corresponding author at: Erasmus MC Room Ee702, Wytemaweg 80, 3015 CN Rotterdam, The Netherlands.
E-mail address: j.essers@erasmusmc.nl (J. Essers).
[1] Present address: Centogene AG, Rostock, Germany.

http://dx.doi.org/10.1016/j.ebiom.2016.09.006

2003; Habashi et al., 2006; Hanada et al., 2007; Huang et al., 2010; Kaijzel et al., 2010; McLaughlin et al., 2006).

Several genes that are involved in cytoskeletal maintenance are mutated in aneurysmal disease. Examples are ACTA2 and MYH11, which function in the contractile apparatus of the smooth muscle cell, and mainly cause thoracic aneurysms when mutated (Guo et al., 2007; Zhu et al., 2006), underscoring the importance of cytoskeleton maintenance and mobility in aneurysmal disease.

The TGF-β pathway plays a relevant role in the etiology of aortic aneurysms. Twenty years ago, the first member of the TGF-β signaling pathway was linked to a genetic vascular disease, after finding mutations in the gene coding endoglin (McAllister et al., 1994). Loss of function mutations in this TGF-β binding protein were described to cause hereditary hemorrhagic telangiectasia type I. This was followed by the discovery of mutations in the receptors TGFβR1 and TGFβR2 (Loeys et al., 2005; Mizuguchi et al., 2004), SMAD3 (van de Laar et al., 2011), the ligands TGFβ2 (Lindsay et al., 2012; Boileau et al., 2012) and TGFβ3 (Bertoli-Avella et al., 2015; Rienhoff et al., 2013). These mutations lead to a spectrum of systemic disorders characterized by aneurysms and other cardiovascular and skeletal features known as Loeys-Dietz syndrome (LDS).

The SMAD (SMA/MAD homology) proteins are important regulators of the TGF-β signaling pathway and function as signaling transducers downstream of TGF-β receptors. The SMAD protein family consists of receptor SMADs (SMAD1–3, SMAD5, SMAD8), the co-effector SMAD4 and inhibitory SMADs (SMAD6 and SMAD7) (Massague, 2012; Massague et al., 2005). Activated SMAD2 and SMAD3 can form heteromeric (pSMAD2/4, pSMAD3/4) complexes in the nucleus where they form transcription-activating complexes capable of inducing or repressing the expression of several genes (Massague et al., 2005; Moustakas and Heldin, 2002) in a cell-type and SMAD complex-dependent manner.

We recently described a genetic disease characterized by aneurysms, dissections and cardiac abnormalities in combination with early-onset osteoarthritis (OA) known as aneurysm-osteoarthritis syndrome (AOS or LDS3; MIM 613795) caused by heterozygous mutations in the *SMAD3* gene (van de Laar et al., 2011). Patients carrying heterozygous *SMAD3* mutations present with extreme clinical variability in cardiovascular disease onset and progression (van de Laar et al., 2012; van der Linde et al., 2012; van der Linde et al., 2013). The exact molecular mechanisms and contributing factors underlying this lack of genotype-phenotype correlation remain to be established, as well as the variable effect that genetic variants of *SMAD3* can have on different tissues. *SMAD3* mutations are suggested to lead to upregulation of the TGF-β pathway in the aortic wall as indicated by nuclear translocated and activated SMAD2 (pSMAD2) (van de Laar et al., 2011). Activated SMAD2 is also seen upon mutational hits in the TGFβR1/2 receptors, or the TGFβ2 ligand (Lindsay and Dietz, 2011; Lindsay et al., 2012; Loeys et al., 2005). Because pSMAD2 is considered to report on activation of the TGFβ pathway, these finding are referred to as the TGFβ paradox, as one would expect that mutations in genes involved the TGFβ pathway would hamper TGFβ signaling (Akhurst, 2012; Massague, 2012). However, it is unclear whether pSMAD2 is a functional marker for the downstream upregulation of the TGFβ pathway. Similarly, mutations in genes involved in build-up and integrity of the ECM lead to an upregulation of the TGF-β signaling pathway and aneurysm formation. For the ECM related gene mutations it is thought that this upregulation is due to release of TGF-β ligand from the ECM, caused by loss of ECM integrity, resulting in ECM remodeling and aortic stiffness (Gillis et al., 2013). It remains to be seen whether the same underlying mechanism is at work when comparing ECM- and TGF-β related gene deficiency in aneurysm formation.

The clinical heterogeneity in AOS patients makes it difficult to study *SMAD3* mutational effects on aneurysm formation. Fortunately, due to the homogenous genetic background, genetically engineered mouse models are useful in pinpointing the specific molecular mechanism leading to disease. *Smad3* knockout animals present with skeletal abnormalities and osteoarthritis (OA) and as such, they have been used as a model to study OA (Yang and Cao, 2001; Li et al., 2009). A cardiovascular phenotype in these animals was overlooked until the recent link of human *SMAD3* mutations and aortic aneurysms was established (Regalado et al., 2011; van de Laar et al., 2011; Ye et al., 2013). Here we describe the cardiovascular phenotype of the *Smad3* knockout mice and reveal the underlying mechanism of aneurysm growth caused by a SMAD3 deficiency.

2. Materials and Methods

2.1. Experimental Animals

Smad3$^{+/-}$ animals were bred into in a C57BL6 background to obtain *Smad3*$^{-/-}$ and *Smad3*$^{+/+}$ experimental animals (backcross 6). The numbers of animals, as well as procedures used, are described in the results section and below, respectively. Animals were housed at the Animal Resource Centre (Erasmus University Medical Centre), which operates in compliance with the "Animal Welfare Act" of the Dutch government, using the "Guide for the Care and Use of Laboratory Animals" as its standard. As required by Dutch law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. An independent Animal Ethics Committee of the Erasmus Medical Center (Stichting DEC Consult) approved these studies (permit number 140–12-05), in accordance with national and international guidelines.

Litter- and gender matched controls were used for each experiment when available.

2.2. Echocardiographic Measurements

Ascending aortic diameter was measured in M-mode, aortic root diameter was measured at the site of the sinus of Valsalva in B-mode. Aortic length was measured as the distance between the sinus of Valsalva and the brachiocephalic trunk. All mice were ventilated and anesthetized with 2.5% isoflurane and echocardiography of the ascending aorta was performed using a Vevo2100 (VisualSonics Inc., Toronto, Canada). Longitudinal echocardiographic measurements of the ascending aorta were performed on 6, 12, 18 and 26 week old *Smad3*$^{-/-}$ and *Smad3*$^{+/+}$ mice ($n = 18$, 8 male and 10 female per genotype).

2.3. Immunohistochemistry

For histological analysis mice were euthanized by CO_2-inhalation. After opening thorax and abdomen, mice were fixed by perfusion fixation through the left ventricle, with PBS and formalin. Organs were weighed and inspected for macroscopic abnormalities. Organs and tissues were fixed in formalin. Aortas were dehydrated through the histokinette processor (Microm), and paraffin embedded, after which 5-μm sections were prepared. Aortas were stained with HE for general pathology, Resorcin-Fuchsin (RF; Elastin von Gieson) for elastin structure, Alcian Blue (AB) to evaluate the extracellular matrix (ECM), and Picrosirius Red (PR) to assess collagen accumulation.

For immunohistochemical analyses, thoracic aortic sections were boiled in 100 mM Tris-HCl [pH 9.0] with 10 mM EDTA at 300 W for 20 min for antigen exposure, and emerged in 3% H_2O_2 in methanol to inhibit endogenous peroxidase for pSmad2, α-SMA, pERK, CD31, MMP, CD3, MAC2 and Ki-67 staining. Slides were first blocked in 5% Protifar in PBS and 0.025% Triton, and incubated with the primary antibodies overnight at 4 °C; Anti-Human Smooth Muscle Actin (1:100 mouse, clone 1A4 Dako), pSmad2 (1:100 monoclonal Rabbit *anti*-pSmad2 (S465|467 (138D4) Cell Signaling), pERK (1:200 Rabbit Polyclonal

Please cite this article as: van der Pluijm, I., et al., Defective Connective Tissue Remodeling in Smad3 Mice Leads to Accelerated Aneurysmal Growth Through Disturbed Downstream TGF-β Si..., EBioMedicine (2016), http://dx.doi.org/10.1016/j.ebiom.2016.09.006