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Original article

Attenuated development of cardiac fibrosis in left ventricular pressure overload by SM16, an orally active inhibitor of ALK5

Kristin V.T. Engebretsen [a,b,c], Kristine Skårdal [b,c], Sigrid Bjørnstad [d], Henriette S. Marstein [a,b,c], Biljana Skrbic [a,b,c], Ivar Sjaastad [b,c], Geir Christensen [b,c], Johannes L. Bjørnstad [a,b,c], Theis Tønnessen [a,b,c,*]

[a] Department of Cardiothoracic Surgery, Oslo University Hospital Ullevål, Oslo, Norway
[b] Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Oslo, Norway
[c] KG Jebsen Cardiac Research Center and Center for Heart Failure Research, University of Oslo, Oslo, Norway
[d] Department of Pathology, Oslo University Hospital Ullevål and University of Oslo, Oslo, Norway

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ABSTRACT

Pressure overload-induced TGF-β signaling activates cardiac fibroblasts (CFB) and leads to increased extracellular matrix (ECM) protein synthesis including fibrosis. Excessive ECM accumulation may in turn affect cardiac function contributing to development of heart failure. The aim of this study was to examine the effects of SM16, an orally active small molecular inhibitor of ALK5, on pressure overload-induced cardiac fibrosis. One week after aortic banding (AB), C57Bl/6J mice were randomized to standard chow or chow with SM16. Sham operated animals served as controls. Following 4 weeks AB, mice were characterized by echocardiography and cardiovascular magnetic resonance before sacrifice. SM16 abolished phosphorylation of SMAD2 induced by AB in vivo and by TGF-β in CFB in vitro. Interestingly, Masson Trichrome and Picrosirius Red stained myocardial left ventricular tissue revealed reduced development of fibrosis and collagen cross-linking following AB in the SM16 treated group, which was confirmed by reduced hydroxyproline incorporation. Furthermore, treatment with SM16 attenuated mRNA expression following induction of AB in vivo and stimulation with TGF-β in CFB in vitro of Col1a2, the cross-linking enzyme LOX, and the pro-fibrotic glycoproteins SPARC and osteopontin. Reduced ECM synthesis by CFB and a reduction in myocardial stiffness due to attenuated development of fibrosis and collagen cross-linking might have contributed to the improved diastolic function and cardiac output seen in vivo, in combination with reduced lung weight and ANP expression by treatment with SM16. Despite these beneficial effects on cardiac function and development of heart failure, mice treated with SM16 exhibited increased mortality, increased LV dilatation and inflammatory heart valve lesions that may limit the use of SM16 and possibly also other small molecular inhibitors of ALK5, as future therapeutic drugs.

1. Introduction

Left ventricular (LV) pressure overload, as seen in patients with aortic stenosis, increases transforming growth factor beta (TGF-β) signaling [1]. This, in turn, activates cardiac fibroblasts (CFB) and leads to myocardial fibrosis due to increased CFB production of extracellular matrix (ECM) i.e. fibrillar collagen [2–4]. Progressive accumulation of fibrillar collagen and other ECM proteins leads to increased myocardial stiffness and will ultimately impair left ventricular (LV) filling, leading to cardiac dysfunction and heart failure [5]. However, no therapeutic strategy has been developed to specifically target development of cardiac fibrosis [6].

TGF-β binds to its receptor type II, recruits and activates receptor type I/activin like kinase (ALK) 5. ALK5 phosphorylates and activates small mothers against decapentaplegic (SMAD) 2/3, which then forms a stable complex with SMAD4 and translocates into the nucleus leading to transcriptional responses [7]. This is known as the primary transduction pathway of TGF-β [8,9], and is believed to play a fundamental role in activation of CFB and ECM deposition [4]. SM16 is an orally active and selective small molecular inhibitor of ALK5, which effectively reduces phosphorylation of SMAD2 [8]. Inhibition of downstream TGF-β signaling by SM16 may potentially lead to reduced activation of cardiac fibroblasts and reduced development of cardiac fibrosis. We have previously examined the early effects of SM16 with focus on the cardiomyocytes in cardiac pressure overload. Our previous study demonstrated that following one week of aortic banding (AB), treatment with SM16 attenuated cardiomyocyte hypertrophy and preserved cardiac function due to improved cardiomyocyte Ca^{2+} handling [10]. However, the effects of SM16 on cardiac fibroblasts are not yet established. Furthermore, our previous study did not examine

* Corresponding author at: Department of Cardiothoracic Surgery, Oslo University Hospital Ullevål, Kirkeveien 166, 0407 Oslo, Norway. Tel.: +47 230 15268; fax: +47 221 17510.
E-mail address: theis.tonnessen@medisin.uio.no (T. Tønnessen).

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the effects of SM16 on cardiac fibrosis since there is no significant accumulation of mature collagens at this early time point, one week after banding of the ascending aorta (AB) in mice [10]. Finding an orally active drug targeting pressure overload-induced cardiac fibrosis is warranted and could prove beneficial to patients with pressure overload-induced cardiac fibrosis and to the large group of patients with heart failure and preserved ejection fraction.

In the present study, we examined the effects of SM16 on development of LV myocardial fibrosis, alterations in other ECM components and in vivo cardiac function in the more chronic phase following induction of pressure overload. To simulate clinical treatment of pressure overload-induced cardiac remodeling, SM16 was given one week after AB in C57Bl/6J mice. Following four weeks AB, mice were examined by in vivo echocardiography and cardiac magnetic resonance (CMR), and the LV by histology and molecular biology. We further evaluated the mechanistic effects of SM16 in primary cultures of neonatal CFB in vitro.

2. Methods

A detailed methods section is included in the Supplementary data.

2.1. Mouse model of pressure overload

All animal experiments were approved by the Norwegian Animal Research Authority (FOTS ID: 3423), which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Anesthesia was induced in 7 week old C57Bl/6J mice with 5% isoflurane gas in a chamber. The mice were intubated using BioLite Intubation Illuminating System (Braintree Scientific, Inc., Braintree, MA), ventilated on Mini-Vent ventilator (Harvard Apparatus, Holliston, MA) with the use of 2.5% isoflurane, and given 0.2 ml buprenorphine (0.3 mg/ml) subcutaneously. AB was performed by placing a ligature around the ascending aorta as previously described [11,12]. Sham operated animals underwent the same procedure but the suture around the aorta was not tightened. Postoperatively the mice were allowed to recover in an incubator at 35 °C. Following one week of surgery, sham and AB mice were randomized to control chow (sham STD and ABSTD) or chow with 0.45 g/kg SM16 (sham SM16 and ABSM16). Four weeks after the operation, all mice were characterized by echocardiography before sacrifice and seven–eight mice in each group were also examined by CMR.

2.2. SM16

SM16 (4-(5-(benso[d] [1,3] dioxol-5-yl)-4(6methylpyridin-2-yl) 1H-imidazol-2-yl)biclyclo[2.2.2]octane-1carboxamide), a 430.5 MW ALK5 kinase inhibitor, was synthesized by The Chemistry Research Solution LLC (George Patterson Blvd., Bristol, PA) and formulated into Purina Rodent chow in the dose of 0.45 g SM16/kg chow by Research Diets (New Brunswick, NJ) as previously provided by the group of Dr. Ling (Biogen Idec, Cambridge, MA) [10,13]. The control group was given the same Purina Rodent chow (Research Diets) without SM16. A concentration of 0.45 g SM16/kg chow has previously been shown to give a plasma concentration of 6.7 ± 2.4 μM and is considered the optimal dose with respect to efficacy and side effects revealed in a dose response study [13]. SM16 is an orally bioavailable kinase inhibitor that binds highly selectively to the ATP-binding site of ALK5 (K_i, 10 nmol/L) and ALK4 (K_i, 1.5 nmol/L) [13]. SM16 has previously been tested against >60 related and unrelated kinases and has shown only moderate off-target effects against pRAF and pP38 and no inhibitory activity against ALK1 and ALK6 [13]. Also, we have previously demonstrated that SM16 has no off target effects on AKT, ERK1/2, I B-α or JNK in cardiomyocytes in vitro [10]. However, SM16 has not previously been used to treat cardiac fibroblasts in vitro. LDH measurements (Pierce LDH Cytotoxicity Assay Kit, Thermo Scientific, Rockford, IL) following treatment of CFB with increasing concentrations of SM16 were done to evaluate possible toxicity. Since treatment with 3- and 4 μM SM16 revealed a minor, but significant, increase in LDH release (Fig. S1), a concentration of 2 μM SM16 was used in the in vitro experiments to avoid potential toxic effects.

2.3. Echocardiography

Mice were anesthetized with 3% isoflurane and 97% oxygen in a chamber. The mice were then placed in a supine position on a heated table breathing 1.75% isoflurane and 98.25% oxygen through a mask. Echocardiographic examinations were carried out by a cardiologist (IS) using VEVO 2100 (Visualsonics, Toronto, Canada) with a 35 MHz transducer and data were analyzed off-line using VEVO 2100 1.1.0 software (Visualsonics). Echocardiographic examination and data analyses were performed blinded to the group of the animals.

2.4. CMR methods and post-processing

The mice were anesthetized using 4% isoflurane and positioned prone in a dedicated animal bed. Anesthesia was maintained by delivery of 1.5% isoflurane, and monitored by observing respiration and heart rate. Body temperature was measured continuously and kept stable at 36–37 °C by a negative feedback system with heated air. Cardiac magnetic resonance imaging was performed on a 9.4 T dedicated small animal system (Agilent Technologies, Inc., Santa Clara, CA). Briefly, 7–9 contiguous short axis slices were acquired using a prospectively triggered multi-frame gradient echo sequence to assess LV volumes and systolic function. In addition, a mid-ventricular short axis phase contrast cine CMR sequence, adapted to assess tissue velocity in mice was acquired and used to evaluate myocardial velocities and strain as previously described [14]. All CMR analyses were performed blinded to the animal group.

2.5. Histology

Mid ventricular 4 μm sections from formaldehyde fixed paraffin embedded hearts (n = 7 in each group) were stained with Masson Trichrome Stain kit (#HT15, Sigma Aldrich, St Louis, MO), Picrosirius Red (PSR) stain kit (#24901 PolyScience, Warrington, PA) Alcian blue (#A5268, Sigma Aldrich) pH 2.5, TUNEL (#G7130, Promega, Madison, WI), pSMAD2 (#3101, Cell Signaling Technology, Danvers, MA) and photographed using 20× or 40× objectives (Axiovision Rel 4.6, Carl Zeiss microscope, GMbH, Jena, Germany). Computer-assisted quantification of myocardial fibrosis in Masson Trichrome and PSR stained sections was performed using a customized Image J macro. Apoptotic cells following TUNEL stain and nuclear staining of pSMAD2 were counted manually. To evaluate heart valve morphology, the hearts were re-embedded longitudinally, and 4 μm coronal sections were stained with hematoxylin and eosin.

2.6. Isolation and stimulation of cardiac fibroblasts

Cardiac fibroblasts were isolated from the LV of 1–3 day old Wistar rats as previously described [11], and stimulated with 10 ng/ml TGF-β (GF111, Millipore, Billerica, MA), or 1 ng/ml CTGF and/or 2 μM SM16 for 48 h. Recombinant human CTGF was a gift from Dr. Mohammed Shakil Ahmed and Dr. Håvard Attramadal, Oslo University Hospital. Recombinant human CTGF was purified from the cell culture medium of transfected HEK293 cells essentially as previously described [15].

2.7. Analyses of collagen content

Left ventricular total collagen content was assessed as hydroxyproline incorporation using high-performance liquid chromatography (HPLC). To distinguish between insoluble and soluble collagen in the LV of ABSTD