Published in final edited form as:

J Mol Cell Cardiol. 2016 January ; 90: 129–138. doi:10.1016/j.yjmcc.2015.12.010.

The TGF- β Pathway Mediates Doxorubicin Effects on Cardiac Endothelial Cells

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Abstract

Elevated ALK4/5 ligands including TGF- β 2 and activins have been linked to cardiovascular remodeling and heart failure. Doxorubicin (Dox) is commonly used as a model of cardiomyopathy, a condition that often precedes cardiovascular remodeling and heart failure. In 7-8-week-old C57Bl/6 male mice treated with Dox we found decreased capillary density, increased levels of ALK4/5 ligand and Smad2/3 transcripts, and increased expression of Smad2/3 transcriptional targets. Human cardiac microvascular endothelial cells (HCMVEC) treated with Dox also showed increased levels of ALK4/5 ligands, Smad2/3 transcriptional targets, a decrease in proliferation and suppression of vascular network formation in a HCMVEC and human cardiac fibroblasts co-culture assay. Our hypothesis is that the deleterious effects of Dox on endothelial cells are mediated in part by the activation of the TGF- β pathway. We used the inhibitor of ALK4/5 kinases SB431542 (SB) in concert with Dox to ascertain the role of TGF- β pathway activation in doxorubicin induced endothelial cell defects. SB prevented the suppression of HCMVEC proliferation in the presence of TGF- β 2 and activin A, and alleviated the inhibition of HCMVEC proliferation by Dox. SB also prevented the suppression of vascular network formation in co-cultures of HCMVEC and human cardiac fibroblasts treated with Dox. Our results show that the inhibition of the TGF- β pathway alleviates the detrimental effects of Dox on endothelial cells in vitro.

Keywords

Cardiomyopathy; TGF- β ; endothelial cells; angiogenesis; anthracyclines

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The authors declare no conflicts of interests in the publication of this manuscript.

1. Introduction

Patients with heart failure often suffer progressive deterioration of cardiac function. This may be due to increased hemodynamic stress leading to pathologic remodeling [1]. Cardiac remodeling is often considered to be a sign of an irreversible cardiac disease that is associated with a poor prognosis. Current treatments primarily slow the functional decline but cannot prevent or reverse the progressive nature of the disease.

A growing body of evidence links elevated levels of TGF- β superfamily ligands to the progression of heart failure. Specifically, increased cardiac levels of activin receptor-like kinase 4 (ALK4¹, ACVR1B) and ALK5 (TGFB1) ligands TGF- β , activins and myostatin have been detected both in patients and animal models of heart failure [2-6]. The levels of these proteins are positively correlated with the severity of the condition [7, 8]. An ALK4/5 ligand binds to a cognate type II receptor, which then forms a complex with a type I receptor (Fig.1). As a result, the serine/threonine kinase activity of the type I receptor is activated to phosphorylate receptor-regulated Smad proteins (R-Smads) Smad2 and Smad3 [9]. By activating Smad2/3 signaling pathways, the ALK4/5 ligands regulate a broad range of processes including inflammation, tissue repair, cell proliferation and cell differentiation [9, 10].

In the cardiovascular system, TGF- β plays a major role in fibrotic cardiac remodeling. Expression of ALK4/5 ligands leads to the activation of fibroblasts in cardiac tissue by promoting myofibroblast differentiation, enhancing the synthesis of extracellular matrix proteins and regulating fibrotic remodeling of the diseased heart in a variety of conditions [11-14]. Inhibition of the TGF- β pathway alleviates left ventricular remodeling, systolic and diastolic dysfunction [15-17]. Although a key role for TGF- β in fibrotic cardiac remodeling has been established, its vascular effects have not been examined. ALK4/5 ligands affect multiple cell types regulating the growth and maintenance of vascular networks [18]. Recent studies show that endothelial cells treated with TGF- β ligands exhibit decreased proliferation, migration and angiogenesis [19-24]. Activin A has been identified as an inhibitor of angiogenesis in vivo [25, 26]. In order to understand the role of the TGF- β pathway in cardiac vascular remodeling, we tested the effects of a small molecular weight inhibitor of the TGF- β pathway on the formation of vascular networks by human cardiac microvascular endothelial cells.

In this study, we have utilized the exposure of mice and human cardiac endothelial cells to doxorubicin (Dox), which is commonly used as a model of cardiomyopathy and [27-29]. Clinical use of this effective anticancer agent has become restricted because of severe cardiac damage and mortality occurring in patients treated with this agent [30, 31]. Anthracycline antibiotics such as Dox cause a dose-related dilated cardiomyopathy when used to treat cancer patients [32]. Cancer survivors represent an important group of patients at risk of premature cardiovascular disease. The cardiovascular complications in former cancer patients are particularly difficult to treat [33]. Dox cardiomyopathy responds poorly

¹ABBREVIATIONS: HCMVEC, human cardiac microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; HCF, human cardiac fibroblasts; Dox, doxorubicin; ALK4/5, activin receptor-like kinase 4/5.

to therapy and often progresses to fatal congestive heart failure [34]. Apoptosis of contractile cells in the heart, cardiomyocytes, has been implicated as a factor contributing to cardiac damage [35, 36]. However, adult cardiomyocytes may be resistant to doxorubicin-induced apoptosis [37, 38]. This study focuses on cardiac microvascular endothelial cells as a target of Dox in the heart.

Unlike other models of cardiomyopathy, Dox cardiomyopathy can be conveniently investigated by in vitro treatments of cardiac cells with Dox. We show that Dox causes profound vascular defects in cardiac tissue, which manifest as decreased capillary density in treated mouse hearts and suppressed formation of vascular networks by human cardiac endothelial cells in vitro. Vascular changes in the treated hearts occurred prior to the development of other manifestations of cardiomyopathy including cardiac fibrosis and deterioration of cardiac function. We detected the increased expression of TGF- β , related factors and their receptors in Dox treated mouse hearts and in cardiac cell cultures. The inhibition of ALK4/5 receptor kinase activity using a small molecular weight inhibitor enhanced vascular network formation by human cardiac endothelial cells treated with Dox, suggesting that this pathway could be a target for the treatment of cardiomyopathy.

2. Materials and Methods

2.1. Reagents and cell culture

Antibodies from Cell Signaling Technologies that were used for western blot and immunocytochemistry include anti-phospho Smad2 (clone 138D4), anti-Smad2 (clone D43B4), anti phospho Smad3 (C25A9), anti Smad3 (C67H9) and anti p21 (clone 12D1). Anti CD31 (Dako clone JC70A), anti Ki67 and anti-cardiac troponin I (Abcam polyclonal antibodies derived in rabbit) were used in immunostaining. Secondary antibodies to mouse and rabbit IgG (H+L) labeled with AlexaFluor 488 and 594 were used for immunocytochemistry (Life Technologies). Secondary antibodies to mouse and rabbit IgG (H+L) labeled with IRDye 800CW and 680LT (LI-COR Biosciences) were used in western blot. Biotinylated isolectin B4 and streptavidin-fluorescein were from Vector Laboratories.

Human cardiac microvascular endothelial cells (HCMVEC), human umbilical vein endothelial cells (HUVEC) and human cardiac fibroblasts (HCF) were purchased from Lonza. Cells were cultured in EGM2-MV, EGM2 and FGM3 complete media, respectively. All experiments were performed between passages six and seven. Cells were passaged using 0.05% Trypsin-EDTA that was neutralized with defined trypsin inhibitor (Life Technologies).

2.2. Doxorubicin treatments of mice

Seven-to-eight-week old male C57BL/6 mice were used in the study. Animals were bred and maintained at the Animal and Veterinary Service facility at the University of Hawaii JABSOM. All procedures described in this study were approved by the University of Hawaii IACUC and conformed to NIH guidelines for care and use of laboratory animals. A sterile solution of Dox in saline was administered intraperitoneally once a week at a dose of 3 mg/kg of body weight (n=24, with 6 Dox weekly injections per animal). Control mice were

injected with saline (n=17). Echocardiography was performed after 6 weeks of Dox injections. Mice were weighed and sacrificed. The hearts were excised, weighed, and processed for histological and immunohistochemical analyses.

2.3. Histology and immunohistochemistry

Hearts were fixed by perfusion with IHC zinc fixative (BD Biosciences) for capillary staining, dehydrated in 30% sucrose in PBS overnight at 4°C and frozen-embedded in OCT compound. Cardiac sections were pre-incubated with permeabilization/blocking buffer (0.1% Triton X-100 and 3% BSA in PBS) for 30 min at room temperature, and then incubated sequentially with biotinylated isolectin B4 and streptavidin-fluorescein. The slides were cover-slipped in Fluoromount-G mounting medium (Southern Biotech) and examined by fluorescence microscopy using Zeiss LSM 510 confocal microscope. The number of capillaries in the field of view was quantified and normalized to the cardiomyocyte area (determined by staining with cardiac troponin I) using ImageJ software. For the collagen staining, hearts were fixed in 4% paraformaldehyde and stained at JABSOM Histology and Imaging core facility using Trichrome Staining Kit (Modified Gomori's) from ScyTek Laboratories (Logan, UT).

2.4. Endothelial cell proliferation and vascular network formation assays

Endothelial cell proliferation was measured by plating 1500 cells per well in 96 well plates. Beginning on day 0, one set of plates was fixed, nuclei were stained with Hoechst 33342 and the plate was imaged on the Cytation3 (Biotek). On days 0, 2, and 4 plates were treated according to the experimental protocol. On days 2, 4 and 6 plates were fixed and nuclei were similarly stained and imaged. The change in cell number or fold change are expressed as a change with respect to the cell counts made on day 0.

Vascular network formation assays were performed as described in [39] with modifications. Specifically, assays consisted of 2500 HCMVEC and 5000 HCF per well plated in 96 well plates using 100µl of endothelial cell media per well. Beginning on day 0, cells were treated every 48 hours according to experimental protocol. At the indicated day, cells were fixed and stained using mouse anti-CD31 and anti-mouse AlexaFluor 488 antibodies. Plates were imaged on the Cytation3 collecting overlapping images using the 4X objective. The images were stitched together using ImageJ and the stitched images analyzed for network length on the Metamorph software platform using the Angiogenesis module.

2.5. Immunocytochemistry

Cells were cultured in 96 well plates and treated according to the experimental protocol. At a given time point, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS. Cells were washed, permeabilized with 0.1% Triton-X 100, blocked with 3% BSA in PBS, and stained overnight at 4°C with antibodies diluted in 3% BSA-PBS-0.05% Tween-20. Primary antibodies were detected with AlexaFluor labeled secondary antibodies diluted in 3% BSA-PBS-0.05% Tween-20. Nuclei were detected with 2 µM Hoechst 33342 in PBS-0.05% Tween-20. Plates were imaged using the Cytation3 (Biotek). The nuclei positive for Hoechst 33342 were counted. The number of nuclei positive for a specific protein was determined using Gen5 Image Plus software.

2.6. Immunoblotting and ELISA

Cells were cultured and treated according to experimental protocol. At a given time point, culture dishes were placed on ice and washed two times with ice cold PBS. Cells were lysed using M-PER (Pierce) with the HALT inhibitor cocktail (Pierce) on ice for 15 minutes. Cell lysates were scraped into the extraction buffer and the insoluble fraction was pelleted at 12,000g for 10 minutes at 2°C. The lysate was assayed for protein concentration (BCA assay, Pierce). Samples were denatured and reduced (4X sample buffer with β -mercaptoethanol, Bio-Rad). The resolved protein was transferred to low fluorescent PVDF using the Biorad Turboblots system. Membranes were blocked with a SeaBlock blocking buffer (ThermoFisher). Primary antibodies were diluted in blocking buffer with 0.1% Tween-20 and incubated overnight at 4°C. Secondary antibodies with the fluorescent tags 800CW and 680LT were diluted in blocking buffer with 0.1% Tween-20 and incubated one hour at room temperature. Membranes were imaged on the Odyssey imaging system (LI-COR Biosciences) using Image Studio software for image capture and analysis. The amount of phosphorylated Smad2 and Smad3 per lane was normalized to the total parent proteins. The amount of total Smad2/3 proteins per lane was normalized to β -actin.

Supernatants of cell culture experiments were harvested for measurements of TGF- β 1, TGF- β 2 and activin A production. Supernatants were clarified by centrifugation at 10,000g for 10 minutes at 4°C and then stored at -80°C. Concentrations of ligands were measured using Quantikine ELISA kits (R&D Systems). Cells were lysed using M-PER extraction reagent (Pierce) per manufacturer's protocol and the protein concentration determined. The amount of ligand secreted in the culture media was normalized to the protein concentration in lysed cells.

2.7. Quantitative PCR and PCR array assays

Co-cultures were conducted using trans-well inserts. 95,000 fibroblasts per insert and 115,000 endothelial cells per well were plated in a 6 well plate format (Corning/Costar). Both the inserts and wells were treated according to experimental protocol. Cultured cells were lysed with TRIzol and the RNA extracted with PureLink Mini Kit (Life Technologies). cDNA was generated (High Fidelity RNA to cDNA, Life Technologies) and real time PCR was performed using human TaqMan primer probe sets (Life Technologies) that span two exons. The threshold counts were normalized to the relative abundance of HPRT1 using the comparative C_T method. Fold changes in gene expression are calculated as the fold change versus control.

Freshly harvested cardiac tissues from control and Dox treated mice used in PCR array assay were quickly immersed into *RNAlater* RNA stabilization reagent and homogenized in TRIzol. TURBO DNA-free Kit (Life Technologies) was used to remove traces of DNA from preparations of total RNA. Gene expression in control and Dox treated hearts was examined using mouse TGF- β /BMP Signaling Pathway RT² Profiler PCR Array (QIAGEN).

2.8. Statistical analysis

Results are expressed as mean $\pm$ Standard Error of Mean (SEM). Statistical analysis was performed initially using normality test. Normally distributed data were subjected to parametric tests, such as *t*-test or analysis of variance. We used one way or two way analysis of variance to compare more than two experimental groups within a data set. Significance was accepted at the $p < 0.05$ level.

3. Results

3.1. Microvascular defects in doxorubicin treated hearts

Mice received weekly injections of Dox or saline for 6 consecutive weeks, and hearts were excised at the end of the treatment course for further analyses. Specifically, reduced capillary density was detected in hearts of mice treated with Dox (**Fig. 2A and B**). Moderately decreased heart weight-to-body weight (HW/BW) ratio was also detected in Dox treated hearts (**Fig. 2C**). We performed analysis of gene expression in mouse hearts after 6 weeks of Dox treatment utilizing mouse BMP/TGF- β pathway PCR array. A partial list of the transcripts that were 2-fold more abundant in Dox-treated hearts is presented in **Table 1**. This group included ALK4/5 ligands and their receptors (Acvr2a, Inhba, Tgfb1, Tgfb1), transcription factors that function in the canonical TGF- β /activin pathway (Smad2, Smad3), known TGF- β target genes (Serpine1, Thbs1, and Cdkn1b), and proteins that participate in storing and/or activation of TGF- β (Ltbp1, Thbs1). Complete lists of up-, downregulated and unchanged by Dox transcripts is presented in *Supplementary Table 1*. Thus, we detected changes in transcript abundance that are indicative of the increased activity of TGF- β pathway, with an associated decrease in capillary density in Dox treated mouse hearts.

3.2. TGF- β pathway inhibitor reduces production of ALK4/5 ligands in doxorubicin treated cardiac cells

Since our in vivo experiments suggested an increased activity of TGF- β /activin pathway in Dox treated hearts, we examined the effects of Dox on the production of these factors by human cardiac cells. The production of activin A and TGF- β isoforms was increased in Dox treated HCMVEC and their co-cultures with cardiac fibroblasts (*Supplementary Fig. 1*). Furthermore, Dox increased Smad2/3 translocation in HCMVEC by approximately 2-fold and augmented Smad2/3 translocation induced by TGF- β 2 in HCMVEC (**Fig. 3A and B**). In order to assess gene expression in co-culture experiments, we utilized tissue culture inserts with HCMVEC and HCF plated in lower and upper chambers, respectively. Specifically, we detected increased expression of genes encoding ALK4/5 ligands and their receptors (Inhba, TGFB1, TGFB2, TGFBR1) as well as Smad2/3 transcriptional target plasminogen activator inhibitor-1 (SERPINE1) in Dox-treated HCMVEC co-cultured with HCF using trans-well inserts (**Fig. 3C**).

We tested the efficacy of ALK4/5/7 receptor kinase inhibitor SB431542 (SB) in preventing effects of TGF- β pathway activation in endothelial cells. SB blocked Smad2/3 phosphorylation and their nuclear translocation in HCMVEC treated with TGF- β 2 and activin A (**Fig. 4** and *Supplementary Fig. 2*) indicating that this pathway is functional in

cardiac endothelial cells and inhibited by SB. Importantly, SB significantly decreased production of activin A and TGF- β 1 proteins in Dox-treated co-cultures.

Similarly, TGF- β pathway inhibitor reversed upregulated expression of genes in HCMVEC that function in TGF- β /activin pathway (**Fig. 3C and D**). Thus, studies utilizing HCMVEC and their co-culture with cardiac fibroblasts corroborated our in vivo findings regarding upregulated production of ALK4/5 ligands in Dox treated mouse hearts

3.3. TGF- β pathway inhibitor enhances proliferation of human endothelial cells treated with ALK4/5 ligands and doxorubicin

Since our results provided evidence for the increased production of ALK4/5 ligands by Dox treated cardiac cells, we tested effects of TGF- β 2 and activin A on HCMVEC proliferation (**Fig. 5**). Both of these ligands severely reduced both the numbers of HCMVEC over 6-day treatment period and percentage of cells positive for the proliferation marker Ki67 ($p < 0.05$). Treatment with SB increased numbers of both untreated and ligand treated HCMVEC and prevented the decrease in Ki67-positive cells that were treated with TGF- β 2 and activin A.

In separate experiments, we examined the proliferation of HCMVEC treated with low (4-16 nM) concentrations of Dox. Dox treatment decreased numbers of endothelial cells in a concentration-dependent manner (**Fig. 6A**). We also detected upregulated expression of p21^{WAF1/CIP1} protein and reduced expression of Ki67 in treated cells (**Fig. 6C and D**). In order to examine if inhibition of proliferation may be due to the production of ALK4/5 ligands by Dox treated endothelial cells, we utilized SB in these experiments. Indeed, SB increased numbers of HCMVEC in all Dox treatment groups suggesting that activation of TGF- β pathway may have contributed to the inhibition of endothelial cell proliferation in this setting (**Fig. 6B**).

3.4. TGF- β pathway inhibitor enhances formation of vascular networks by doxorubicin treated human endothelial cells

Microvascular defects observed in cardiomyopathic hearts of Dox-treated mice prompted us to examine the direct action of this agent on vascular network formation. We utilized co-culture systems of HCMVEC or HUVEC with human cardiac fibroblasts. These systems develop intricate vessel-like structures after 5 to 7 days in co-culture (**Fig. 7A**). Doxorubicin potently inhibited the formation of vascular networks in these co-culture systems. Quantitative evaluation of captured images demonstrated that Dox suppressed vascular network formation and reduced the number of endothelial cells in co-cultures by 70% and 85%, respectively (**Fig. 7A and B**). Similar results were obtained with HUVEC/HCF co-cultures (data not shown). Further analysis has shown that Dox did not cause acute endothelial cell toxicity or apoptotic cell death within the range of concentrations used in these experiments, but caused severe inhibition of HCMVEC proliferation in treated co-cultures (data not shown). SB enhanced vascular network formation in HCMVEC/HCF co-cultures treated with Dox (**Fig. 7A and B**). Similarly, endothelial cell number was significantly increased by SB in both intact and Dox treated co-cultures (**Fig. 7C**). Similar results were obtained with a structurally different ALK4/5 inhibitor SB525334 (data not shown). It is important to note that SB did not increase numbers of fibroblasts in both

control and Dox treated co-cultures (**Fig. 7D**). Thus, Dox suppressed formation of vessel-like networks in HCMVEC/HCF co-cultures, and its inhibitory effect was alleviated by the TGF- β pathway inhibitor.

4. Discussion

Dox caused microvascular defects in the hearts of treated mice and suppression of the formation of vessel-like structures in HCMVEC co-cultured with HCF. We detected changes in gene expression that were indicative of the increased activity of the TGF- β pathway. Incubation of HCMVEC with either TGF- β 2 or Activin A inhibited proliferation while a TGF- β pathway inhibitor abolished the defect in endothelial cells. Similarly, Dox inhibited HCMVEC proliferation but a TGF- β inhibitor alleviated this effect of Dox and enhanced formation of vessel-like structures in Dox treated co-cultures.

Decreased cardiac capillary density is considered to be a sign of microvascular deficit [40], which, in the case of Dox treatment, developed before other known markers of Dox cardiomyopathy including depressed cardiac function and myocardial fibrosis (data not shown). Reduced capillary density preceded the development of tissue hypoxia in renal cortex of Dox treated animals [41]. Microvascular deficit induced by the trapping of VEGF in myocardial tissue has been shown to trigger cardiac remodeling and failure while restoring VEGF levels reversed remodeling and rescued compromised ventricular function [40]. Microvascular deficiency is a crucial factor that causes hypertrophic, ischemic and cardiomyopathic conditions to progress towards a decompensated functional state. Strategies aimed at the activation of angiogenesis delay or prevent decompensation of cardiac function [42-46]. Thus, our data and literature considerations suggest that endothelial defects caused by Dox in the heart may contribute to the microvascular deficiency and development of cardiomyopathy in treated hearts.

4.1. Endothelial cell as a target of doxorubicin in the heart

Several cardiac cell types, including cardiomyocytes, cardiac progenitor cells, and endothelial cells have been considered as targets of doxorubicin in the heart that contribute to the development of cardiomyopathy [28, 35, 37, 47]. Huang et al. [28] have previously reported decreased capillary density in hearts treated with Dox. Interestingly, a study performed on hair-follicle vascular network found that endothelial cells were much more vulnerable to the damaging effects of Dox than hair follicle stem cells [48].

Apoptosis was considered to be the primary mechanism of endothelial injury by Dox [49, 50]. Indeed, our previous study supported this mechanism of Dox induced endothelial cell injury [51]. Concentrations of Dox used in these earlier studies and many other studies induced a strong apoptotic response that resulted in death of most endothelial cells in culture within several days. Such an apparent acute endothelial cell injury does not occur in patients treated with Dox [32]. Indeed, exposure of patients to anthracyclines often results in delayed cardiovascular toxicity, which manifests itself as left ventricular dysfunction that ultimately progresses to congestive heart failure [32, 33, 52, 53]. In addition, clinical follow-up studies detected chronic vascular inflammation and early atherosclerotic lesions in survivors of

Hodgkin lymphoma and osteosarcoma treated with Dox and other chemotherapeutic agents [54, 55].

In the current study we used concentrations of Dox that were comparable to its plasma levels in animals after the injection of this agent [56], and did not detect apoptotic or necrotic endothelial cell death markers (data not shown). Our earlier studies with Dox showed that it is avidly and rapidly taken up by the cultured cells [57]. In fact, concentration of Dox in cultured cells typically exceeds its levels in culture media by 50 to 100-fold [58]. Estimated cellular concentrations of Dox in our in vitro experiments are similar to cardiac levels of this agent in animals received a clinically relevant dose of Dox [59-61]. Thus, we believe that the present study was conducted using Dox concentrations that are commonly obtained during clinical use of the agent, and the types of endothelial defects observed at these concentrations of Dox may therefore be clinically relevant.

Another previously suggested mechanism of endothelial damage by Dox involves either decreased expression of VEGF by treated tissues or VEGF receptor 2 (VEGFR2) by exposed endothelial cells [62-64]. At the concentrations that were used in the current study, Dox did not significantly change production of VEGF by HCMVEC/HCF co-cultures or expression of VEGFR2 by HCMVEC (data not shown). The endothelial cell growth media used in our experiments with mono-cultures and co-cultures was supplemented with human recombinant VEGF. We show that Dox significantly decreased numbers of endothelial cells, both in mono-cultures and co-cultures, as a result of inhibition of proliferation in the presence of VEGF. Specifically, it decreased expression of Ki67, a cell proliferation marker, and increased abundance of p21^{WAF1/CIP1} protein in treated endothelial cells. Induction of p21^{WAF1/CIP1} and cell cycle arrest are known consequences of the p53 transactivation program. Dox has been previously shown by us and others to induce transcriptional activation of p53 in endothelial cells [49, 65]. However, Dox caused neither p53 transactivation nor upregulation of p53 target genes in HCMVEC at low nanomolar concentrations used in the current study (data not shown).

4.2. Endothelial defects due to the increased activity of TGF- β pathway

Our initial observation of increased expression of genes that function in TGF- β pathway in Dox treated hearts was supported by our in-vitro experiments with human cardiac cell types. Specifically, we found that Dox increased production of ALK4/5 ligands by co-cultures of HCMVEC/HCF, and increased expression of ALK4/5 ligands and known TGF- β target genes. It has been known that TGF- β inhibits proliferation of cells of epithelial origin [66, 67]. We show in this study that ALK4/5 ligands, TGF- β 2 and activin A, suppress proliferation of human cardiac endothelial cells. Several studies have previously reported inhibition of proliferation and migration by ALK4/5 ligands in endothelial cells of other origins [19, 20, 24]. Therefore, it is plausible that inhibition of ALK4/5 receptor signaling will enhance formation and improve maintenance of microvascular networks via direct effects on endothelial cell proliferation and migration. Indeed, suppression of TGF- β signaling by ALK4/5/7 receptor kinases inhibitor SB431542 [68] in endothelial cells led to the increased vascular sprouting by immortalized mouse endothelial cells and HUVEC in vitro [23, 69], preserved microvascular networks in such conditions as radiation injury and

chronic kidney disease [70, 71] and increased proliferation of human embryonic stem cell derived endothelial cells [72, 73]. Likewise, SB431542 facilitated direct conversion of embryonic and adult cardiac fibroblasts into induced cardiomyocytes [74]. Increased efficiency of the fibroblast-to-cardiomyocyte reprogramming by SB431542 may potentially enhance regenerative capacity of cardiac muscle.

Effects of inhibition of TGF- β signaling in cardiac endothelial cells have not been reported before. We show here that SB431542 prevents the suppression of HCMVEC proliferation caused by ALK4/5 ligands. In addition, this inhibitor enhanced formation of vessel-like structures in control co-cultures and alleviated the inhibition of proliferation as well as suppression of formation of vessel-like networks by HCMVEC induced by Dox. These effects are likely due to the blocking the effects of ALK4/5 ligands on endothelial cells in both control and Dox treated co-cultures. In a co-culture, endothelial cells are exposed to higher concentrations of TGF- β and activin that are produced by fibroblasts, and Dox further increases production of these factors in this system. Increased levels of ALK4/5 ligands contribute to the suppression of endothelial cell proliferation in co-culture while SB431542 blocks their effects on endothelial cells to enhance their proliferation and vascular network formation. Importantly, in addition to the inhibition of signaling through ALK4/5 receptors by preventing Smad2/3 phosphorylation and nuclear translocation, SB decreased production of activin A and TGF- β 1 by human cardiac cells. This effect on synthesis may further inhibit TGF- β pathway activity, interrupting the auto-induction loop whereby TGF- β 1 and activins enhance their own expression [75-78].

Interestingly, SB431542 increased proliferation of endothelial cells but decreased numbers of fibroblasts in control co-culture experiments. Indeed, TGF- β is known to increase proliferation of fibroblastic cells and cancer cells that have inactivating mutations in the canonical TGF- β pathway [79-82]. TGF- β may contribute to the tumor growth and progression by enhancing fibroblast proliferation and their migration of into the tumor [83]. Cancer associated fibroblasts produce increased amounts of growth factors and cytokines that enhance both proliferation of tumor cells and angiogenesis, and increase resistance of cancer cells to chemotherapeutic agents [84]. In addition, TGF- β ligands are potent inducers of the epithelial-to-mesenchymal transition in transformed cells, leading to their enhanced migration and invasiveness [85]. Inhibition of TGF- β pathway with SB431542 sensitized resistant cancer cells to chemotherapy and suppressed cancer cell proliferation, migration and metastases [86, 87]. Thus, using SB431542 or other TGF- β pathway inhibitor under such circumstances to supplement the chemotherapy with Dox may not only enhance its anticancer efficiency but also protect the heart from the Dox toxicity.

4.3. Conclusions

Small molecular weight inhibitors of TGF- β pathway have been reported to alleviate fibrosis [88] and peritubular capillary defects [89] in Dox induced nephropathy. The results of the present study suggest that Dox causes increased expression of ALK4/5 ligands and vascular remodeling in treated mouse hearts, with reduced myocardial capillary density. This type of microvascular remodeling has been described in other types of cardiomyopathies, including diabetic and idiopathic dilated cardiomyopathies [43, 90]. Myocardial microvascular

deficiency promotes the progression of cardiac disease. Results of this study suggest inhibition of TGF- β pathway as a plausible strategy aimed at the preservation and enhancement of myocardial capillary networks and prevention of microvascular remodeling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health 2P20RR016467-09A1 INBRE II grant. The funding source had no involvement in study design, the collection, analysis and interpretation of data, the writing of the report, and the decision to submit the article for publication.

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Highlights

- Doxorubicin causes decreased capillary density in treated mouse hearts
- Doxorubicin increases expression of ALK4/5 ligands in cardiac cells
- ALK4/5 ligands suppress cardiac endothelial cell proliferation
- Doxorubicin inhibits cardiac endothelial cells proliferation and angiogenesis
- ALK4/5/7 kinase inhibitor alleviates the anti-angiogenic effect of doxorubicin

Figure 1.
Scheme of ALK4/5 ligands and their receptors

Figure 2. Microvascular defects in doxorubicin treated mouse hearts

Mice received weekly intraperitoneal injections of Dox (3 mg/kg) or saline for six weeks.

(A) Cardiac tissue sections were stained for capillaries using isolectin B4 (IL-B4, green) and counterstained for cardiac troponin I (blue). (B) Quantitative analysis of capillary density is presented as number of capillaries per field of view normalized to myocardial (cardiac troponin I positive) area (n=3 hearts for saline group; n=3 hearts for Dox treated group). (C) Heart weight (HW) and body weight (BW) were taken at the end of the Dox treatment period to determine HW/BW ratio (n=3 for saline group; n=4 for Dox treated group).

*p<0.05 as compared with control. Scale bar, 100 μ m.

Figure 3. Enhancement of Smad2/3 nuclear localization and TGF- β pathway by Dox

(A) HCMVEC cultures were treated with 16 nM Dox for 72 hours. Cultures were starved for six hours, then treated with 0.3 ng/ml TGF- β 2 and stained for Smad2/3 and CD31. (B) Smad2/3 nuclear localization measured as the percentage of nuclei that are positive for Smad2/3. (C) HCMVEC and HCF were plated in the lower and upper wells of a tissue culture insert, respectively, and treated with 32 nM Dox and 1 μ M SB431542 (SB) for 48 hours. Gene expression in HCMVEC was examined using quantitative PCR and TaqMan gene expression assays. Expression of HPRT1 gene was used as an endogenous control. (D) HCMVEC and HCF were plated in a mixed co-culture and treated with 32 nM Dox and 1 μ M SB for 72 hours. Concentrations of TGF- β 1 and activin A proteins in culture media were examined using ELISA and normalized to total cell protein. * and #, $p < 0.05$ as compared with control and Dox groups, respectively. Scale bar, 100 μ M.

Figure 4. Inhibition of Smad2/3 phosphorylation and nuclear translocation in HCMVEC by SB431542

(A) HCMVEC were cultured in complete EGM2-MV media, starved overnight, treated with 3 ng/ml TGF- β 2 for 60 minutes and processed for immunoblotting. SB431542 (SB) was added to HCMVEC cultures 60 min prior to TGF- β 2 treatment. (B) HCMVEC were treated with TGF- β 2 and SB as described in (A) and stained for Smad3 (red). Nuclei were counterstained with Hoechst 33342 (blue). (C) Statistical analysis of Smad3 nuclear translocation images. * and #, $p < 0.05$ as compared with control and TGF- β 2, respectively. Scale bar, 50 μ m.

Figure 5. TGF- β pathway inhibitor enhances proliferation of HCMVEC treated with ALK4/5 ligands

HCMVEC were treated with 10 ng/ml activin A (**A**), 0.3 ng/ml TGF- β 2 (**B**) and nuclei were stained at indicated time points with Hoechst 33342 for the total cell number evaluation. SB431542 (SB) was present at 1 μ M concentration. * and #, $p < 0.05$ as compared to control and ligand only treated groups, respectively. (**C**, **D**) HCMVEC cultures were treated with Dox and SB as described and stained for CD31 (green) and Ki67 (red). Images were acquired and analyzed using Cytation 3 imaging system. * $p < 0.05$ for SB(+) groups as compared to the corresponding SB(-) groups. Scale bar, 100 μ m.

Figure 6. Doxorubicin inhibits proliferation of HCMVEC

(A) HCMVEC were treated with the indicated concentrations of Dox and nuclei were stained with Hoechst 33342 for the total cell counts at the indicated time points. *, $p < 0.05$ as compared with control. (B) HCMVEC were treated with the indicated concentrations of Dox and 1 μ M SB431542 (SB) for 6 days. Nuclei were stained with Hoechst 33342 for the total cell counts per well. *, $p < 0.05$ as compared to SB(-) group. (C and D) HCMVEC were treated with the indicated concentrations of Dox and stained for CD31 (green) and Ki67 or p21 (red). Images were acquired and analyzed using Cytation 3 imaging system. *, $p < 0.05$ as compared with control. Scale bar, 100 μ m.

Figure 7. TGF- β pathway inhibitor enhances formation of vessel-like structures by doxorubicin treated HCMVEC

(A) HCMVEC/HCF co-cultures (2,500 HCMVEC and 5,000 HCF) were treated with 32 nM Dox and 1 μ M SB431542 (SB), and stained at day 7 for CD31 to visualize the vessel-like networks. (B) Day 7 network images were analyzed using Metamorph Angiogenesis module software and presented as total length of vessel-like structures per well. (C and D) Day 3 co-cultures were stained for CD31 and Hoechst 33342 to visualize HCMVEC and HCF. Numbers of endothelial cells and fibroblasts per well were quantified using Gen5 Image Plus software. * and #, $p < 0.05$ as compared to untreated control and Dox only treated groups, respectively. Scale bar, 500 μ m.

Table 1TGF β /activin pathway genes upregulated in doxorubicin treated mouse hearts

Gene Symbol	Gene Full Name
Acvr2a	activin A receptor, type IIA
Cdkn1b	cyclin-dependent kinase inhibitor 1B
Inha	inhibin, alpha
Inhba	inhibin, beta A
Ltbp1	latent transforming growth factor beta binding protein 1
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1
Smad2	SMAD family member 2
Smad3	SMAD family member 3
Smurf1	SMAD specific E3 ubiquitin protein ligase 1
Tgfb1	transforming growth factor, beta 1
Tgfb1r1	transforming growth factor, beta receptor 1
Thbs1	thrombospondin 1