Published in final edited form as:

Cardiovasc Pathol. 2013 January ; 22(1): 54–57. doi:10.1016/j.carpath.2012.03.004.

Canonical Wnt/ β -catenin signaling in epicardial fibrosis of failed pediatric heart allografts with diastolic dysfunction

Bo Ye, MD and PhD¹, Yao Ge, MD¹, Gregory Perens, MD², Longsheng Hong, MD³, Haodong Xu, MD and PhD¹, Michael C. Fishbein, MD³, and Faqian Li, MD and PhD^{1,4}

¹Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, NY 14642, USA

²Department of Pediatrics, Mattel Children's Hospital at UCLA

³Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095, USA

Abstract

Background—Failed pediatric heart allografts with diastolic dysfunction exhibit severe epicardial fibrosis. The molecular mechanism underlying this process is poorly understood. Canonical Wnt/ β -catenin signaling plays an important role in epithelial-mesenchymal transition and is implicated in fibrosing diseases. In this study, we tested the hypothesis that canonical Wnt/ β -catenin signaling is activated in epicardial fibrosis of end-stage dysfunctional pediatric allografts.

Methods—Fourteen explanted heart grafts of 12 patients who had undergone 14 heart transplantations were used for immunohistochemical staining of β -catenin and its nuclear binding partners, T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcriptional factors. Fourteen age-matched native hearts from patients who undergone first heart transplantation without evidence of epicardial fibrosis were used as controls.

Results and Conclusions—Epicardial fibroblasts from explanted allografts demonstrated nuclear accumulation of β -catenin. These cells also showed nuclear positivity for TCF-4. No TCF-3 expression was present in the epicardium. TCF-1 and LEF-1 were observed in lymphocytes, but not in other cell types of the epicardium. These findings suggest an association between canonical Wnt/ β -catenin signaling and epicardial fibrosis of failed pediatric heart allografts. Should activation of this pathway be shown to be causal to epicardial fibrosis in this setting, then inhibition of this pathway may help to prevent this devastating process.

Keywords

Heart transplant; Pediatric; Epicardium; Fibrosis; Allograft; Heart failure; Wnt signaling; LEF/TCF; β -Catenin

© 2012 Elsevier Inc. All rights reserved.

⁴**Corresponding Author:** Faqian Li, MD, PhD, Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, 601 Elmwood Avenue, Box 626, Rochester, NY 14642, USA. Telephone: 585-2764651. Fax: 585-273-3637. Faqian_Li@urmc.rochester.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

There is no conflict of Interest to disclose.

Introduction

Heart transplantation is an effective treatment option for children with heart failure due to congenital and genetic heart abnormalities. However, the long-term survival of the graft is limited by chronic rejection as treatments for acute rejection are significantly improved. Coronary artery vasculopathy (CAV) considered as a form of chronic rejection is the major cause of graft failure. We found epicardial fibrosis which often accompanied CAV contributed to diastolic dysfunction of end-stage cardiac allografts. The underlying mechanism leading to epicardial fibrosis is not known. Wnt signaling pathway regulates epithelial-mesenchymal transformation during development and has been implicated in fibromatosis. Further investigations have revealed that β -catenin is activated during wound healing, myocardial infarction, and pulmonary fibrosis. It is not clear whether this pathway is also involved in epicardial fibrosis of end-stage cardiac allografts.

In canonical Wnt signaling, Wnt ligands bind to frizzled family membrane receptors and their co-receptors, LRP5/6, to regulate β -catenin stability. In the cytoplasm, β -catenin interacts with adenomatous polyposis coli, glycogen synthase kinase-3 β (GSK-3 β), and axin, to form the so called destruction complex. GSK-3 β regulates cytoplasmic β -catenin levels via phosphorylation and ubiquitin-mediated proteasome degradation of β -catenin. GSK-3 β is constitutively active in resting cells and is primarily regulated by inactivation. The interaction of Wnt ligands with their receptor and co-receptor leads to the inactivation of GSK-3 β by phosphorylating its serine 9. When GSK-3 β activity is inhibited, β -catenin is less phosphorylated and thus becomes stabilized leading to an increase in its cytoplasmic levels. Subsequently, β -catenin enters the nucleus to form a complex with T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcriptional factors, activating transcription of target genes such as c-myc and cyclin D1. Although there is only one β -catenin gene in mammals, four TCF/LEF transcriptional factors have been identified in human. These nuclear partners show differential expression during development and in a variety of human diseases.

In this study, we performed detailed analysis of the morphologic pattern and distribution of epicardial fibrosis in end-stage cardiac allografts. More importantly, we examined the expression of β -catenin and its nuclear binding partners: LEF-1, TCF-1, TCF-3 and TCF-4 in the epicardium. Epicardial fibrosis in end-stage cardiac allografts involved either epicardial surface or underlying adipose tissue or both. Fibroblasts in epicardial fibrosis demonstrated β -catenin nuclear accumulation, a hallmark of canonical Wnt signaling activation. Interestingly, only TCF4, one of 4 LEF/TCF family members, was detected in epicardial fibroblasts. Our results suggest that canonical Wnt/ β -catenin signaling is associated with epicardial fibrosis of failed pediatric heart allografts. Should activation of this pathway be shown to be causal to epicardial fibrosis with an animal model, then inhibition of this pathway may help to prevent this devastating process.

Material and Method

The epicardium of fourteen heart allografts explanted from 12 patients during heart transplantations at UCLA Medical Center from 1998 to 2007 were used in this study. The clinical status, pathology, echocardiographic, and catheterization data of these patients were reviewed and reported previously. Seven patients were transplanted for cardiomyopathy, 2 for hypoplastic left ventricle syndrome, 1 for Tetralogy of Fallot, and 1 for common AV canal. Fourteen age-matched native hearts from patients who undergone first heart transplantation without evidence of pericardial fibrosis were used as controls. Heart transplantation was the first heart surgery for these 14 patient. Epicardial fibrosis was evaluated on the epicardial surface and subepicardial fat, and graded as mild (focal),

moderate (multifocal) and severe (diffuse). Similarly, Epicardial inflammation was graded as mild (focal), moderate (multifocal) and severe (diffuse).

One representative block of the epicardium from each heart was selected to cut 4 μ m sections for immunohistochemical staining as previously described . Antigen retrieval was performed in EDTA buffer (pH: 9.0) for β -catenin (1:500, Sigma C2206), LEF1 (1:100, Cell Signaling 2230), TCF1 (1:100, Cell Signaling 2203), TCF3 (1:250, Epitomics EPR2031), TCF3/4 (1:200, Cell Signaling 05-512), TCF4 (1:400, Millipore, 04-1080), and TCF 4 (1:200, Cell Signaling 2569) by heating to 99°C for 20 minutes with a PT Link system from Dako (Carpinteria, CA). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide and non-specific binding was blocked with 10% non-immune goat serum (Invitrogen, Carlsbad, CA). Primary antibodies were incubated in a moisturized chamber at 4°C overnight. The signal was amplified with the Histostain-SP Kit (Invitrogen, Carlsbad, CA) according to manufacture's instruction. Briefly, slides were incubated with biotinylated secondary antibody for 10 minutes at room temperature, and then followed by streptavidin-horseradish peroxidase conjugate for 10 minutes. Finally, peroxidase activity was detected with DAB substrate (Dako) Hematoxylin was used as the counterstain. Negative controls were incubated with appropriate serum instead of primary antibody under the same conditions. Skin and basal cell carcinoma were used for positive control of TCF3.

Result

The epicardium from native hearts had a thin layer of fibrous tissue on the surface (Figure 1A). Variable amounts of adipose tissue were present between the epicardial surface and the myocardium. No significant inflammatory infiltrate was observed in native hearts. Rare scattered fibroblasts were present. The clinical, echocardiographic, and catheterization data of pediatric patients with failed heart allografts at the time of the listing for heart transplantation were previously reported . These failed allografts had severe diastolic dysfunction with relatively preserved systolic function. The epicardium of these explanted allografts had significant fibrosis involving the epicardial surface, subepicardial fat, or both (Figure 1B and 1C). Most cases (10 of 14) contained severe fibrosis involved both epicardial surface and underlying fat. Two cases had moderate epicardial surface fibrosis, but mild subepicardial fat fibrosis. One case showed mild surface and moderate subepicardial fat fibrosis, and the one other case demonstrated moderate surface and severe subepicardial fat fibrosis. There was mild to severe epicardial inflammation, predominantly as lymphoid aggregates, in all failed allografts (Figure 1B and 1D). Among 11 cases with severe epicardial fibrosis, 3 cases also had severe inflammation. The degree of epicardial inflammation was not apparently associated with the severity of epicardial fibrosis in other cases. Four cases with severe epicardial fibrosis revealed only mild epicardial inflammation. On the other hand, 2 cases with mild epicardial fibrosis demonstrated moderate epicardial inflammation. Three cases with severe epicardial fibrosis contained moderate epicardial inflammation while 1 case with moderate epicardial fibrosis had moderate epicardial inflammation.

In epicardial fibrosis of failed allografts, there were 2 main types of cells: fibroblasts and lymphocytes. These cells were not easily found in native non-fibrotic epicardium. Both lymphocytes and fibroblasts in fibrotic epicardium demonstrated nuclear positivity for TCF4 (Figure 1F) and β -catenin (Figure 1H and 1I). TCF4 specific antibodies from Cell signaling and Millipore gave similar staining pattern. In addition, an antibody reactive with both TCF3 and TCF4 from Cell signaling also showed identical result. However, no TCF-3 expression was detected in any cells of the epicardium from either native hearts or allografts. As a positive control (not shown), epidermis and skin adnexal structure as well as basal cell carcinoma were positive for TCF3 as previously reported . Lymphocytes in fibrotic

epicardium demonstrated nuclear positivity for both TCF1 (Figure 1J) and LEF1 (Figure 1K). However, fibroblasts were negative for these 2 TCF/LEF family members (Figure 1J and 1K).

Discussion

Our previous study revealed that end-stage pediatric cardiac allografts showed significant epicardial fibrosis. These epicardial changes correlated with the restrictive hemodynamics of these grafts assessed for relisting. Morphologic analysis revealed that fibroblastic proliferation and collagen deposition involved epicardial surface and underlying epicardial adipose tissue. These fibroblasts demonstrated nuclear accumulation of β -catenin, a hallmark of canonical Wnt signaling activation. The nuclear translocation of β -catenin is involved in epithelial-mesenchymal transformation, which is an initial and key step in fibrosing processes such as pulmonary fibrosis and myocardial infarction repair.

Patients with pericardial diseases such as constrictive pericarditis or open heart surgery to correct structural defects can develop pericardial adhesions with thicken epicardium. Most of our patients did not have open cardiac surgery or pericardial fibrosis in their native hearts before heart transplantation. Whether Wnt/ β -catenin signaling is also activated in these conditions is clinically relevant, but undetermined. Answering this question will help clarify whether Wnt/ β -catenin activation is unique to epicardial fibrosis of cardiac allograft or a common repair response to cardiac surgery.

There are 4 β -catenin nuclear partners of TCF/LEF family. In adults, Tcf-1 and Lef-1 are mainly expressed in lymphoid tissues by Northern blotting. Lef-1 could not compensate the loss of Tcf-1 in T-cells as global deletion of Tcf-1 affects T-cell differentiation and maturation. In pediatric heart allografts, both Tcf-1 and Lef-1 were present in epicardial lymphoid infiltrates. In normal hearts, Tcf-1 or Lef-1 can not be detected by Western or Northern blotting. We did not find Tcf-1 and Lef-1 expression in any cells of the epicardium from either native hearts or cardiac allografts except in lymphocytes. Tcf-3 is ubiquitously expressed during early mouse development, but gradually disappears after E7.5 and becomes undetectable by in-situ hybridization after embryonic day (E)10.5. Similarly, we did not observe Tcf-3 expression in either native hearts or cardiac allografts in this study by immunohistochemistry. Tcf-4 is first expressed at E10.5 by in-situ hybridization and mainly found in di- and mes-encephalon and the intestinal epithelium during mouse development. In adult mice, Tcf-4 has wider tissue expression than other TCF/LEF family members. TCF4 is abundant in brain, but is also detected in other tissues by Northern blotting. Another investigation has revealed wider and higher expression of Tcf-4 in adult mice with highest levels observed in the liver, an endodermally derived organ. Additionally, TCF4 is detected in intestines and mammary glands by immunohistochemistry. In this study, we found TCF-4, but not other TCF/LEF family members, was expressed in epicardial fibroblasts of failed pediatric heart allografts. This indicates that TCF4 is the only nuclear partner of β -catenin in cardiac fibroblasts in the TCF/LEF family.

Interstitial and perivascular lymphocytic infiltration in the myocardium after transplantation is a major morphologic feature of acute cellular rejection, which is graded dependent on the pattern, distribution, and severity of lymphoid infiltrates. However, purely endocardial lymphoid aggregation is generally considered as so-called "Quilty lesions", but not rejection. Epicardial lymphoid infiltrates that can be associated with and may mimic acute cellular rejection, are occasionally observed in endomyocardial biopsy for cardiac transplant rejection evaluation, and frequently seen in autopsy cases. However, Quilty lesions exhibit morphologic and immunophenotypic features which are distinguishable from rejection-associated infiltrates. Although we showed the nuclear positivity of β -catenin, TCF4,

TCF-1 and LEF-1 in epicardial lymphocytes of explanted pediatric allografts, these markers were also expressed in lymphocytes of benign lymph nodes or tonsils. All failed heart allografts had variable amounts of lymphoid infiltrates in the epicardium. However, we did not observe apparent association between the degree of lymphoid infiltrate and severity of epicardial fibrosis in failed allografts.

In summary, we found that fibroblast in epicardial fibrosis of failed pediatric heart allografts showed nuclear accumulation of β -catenin together with its nuclear partner, TCF4. Nuclear translocation of β -catenin is a cardinal sign of canonical Wnt signaling. This suggests that the canonical Wnt pathway is activated in epicardial fibrosis of failed pediatric heart transplants. Recent advancements in targeting canonical Wnt signaling have produced promising therapeutic options to inhibit its activity. If Wnt activation is proven causal to epicardial fibrosis with an animal model, then Wnt inhibitors can be explored for treatment.

Acknowledgments

Michael C. Fishbein is supported by The Piansky Family Trust. Faqian Li has a Grant-in-Aid award (10GRNT4460014) from the American Heart Association Greater River Affiliate and the Lawrence J. and Florence A. DeGeorge Charitable Trust. Haodong Xu is supported by grant number K08 HL088127 from the National Institutes of Health (NIH).

References

- [1]. Kirk R, Edwards LB, Aurora P, Taylor DO, Christie J, Dobbels F, et al. Registry of the International Society for Heart and Lung Transplantation: Eleventh Official Pediatric Heart Transplantation Report--2008. The Journal of Heart and Lung Transplantation. 2008; 27:970–7. [PubMed: 18765188]
- [2]. Pahl E, Naftel DC, Kuhn MA, Shaddy RE, Morrow WR, Canter CE, et al. The impact and outcome of transplant coronary artery disease in a pediatric population: a 9-year multi-institutional study. J Heart Lung Transplant. 2005; 24:645–51. [PubMed: 15949722]
- [3]. Perens G, Li F, Meier S, Kaur R, Alejos JC, Fishbein M. Clinico-pathologic findings in end-stage pediatric heart transplant grafts. Pediatric Transplantation. 2009; 13:887–91. [PubMed: 19017288]
- [4]. Alman BA, Li C, Pajerski ME, Diaz-Cano S, Wolfe HJ. Increased beta-catenin protein and somatic APC mutations in sporadic aggressive fibromatoses (desmoid tumors). Am J Pathol. 1997; 151:329–34. [PubMed: 9250146]
- [5]. Cheon SS, Cheah AY, Turley S, Nadesan P, Poon R, Clevers H, et al. beta-Catenin stabilization dysregulates mesenchymal cell proliferation, motility, and invasiveness and causes aggressive fibromatosis and hyperplastic cutaneous wounds. Proc Natl Acad Sci U S A. 2002; 99:6973–8. [PubMed: 11983872]
- [6]. Blankesteyn WM, van Gijn ME, Essers-Janssen YP, Daemen MJ, Smits JF. Beta-catenin, an inducer of uncontrolled cell proliferation and migration in malignancies, is localized in the cytoplasm of vascular endothelium during neovascularization after myocardial infarction. Am J Pathol. 2000; 157:877–83. [PubMed: 10980127]
- [7]. Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, et al. Aberrant Wnt/ β -Catenin Pathway Activation in Idiopathic Pulmonary Fibrosis. Am J Pathol. 2003; 162:1495–502. [PubMed: 12707032]
- [8]. Kishida S, Yamamoto H, Ikeda S, Kishida M, Sakamoto I, Koyama S, et al. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem. 1998; 273:10823–6. [PubMed: 9556553]
- [9]. Miller JR, Moon RT. Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes Dev. 1996; 10:2527–39. [PubMed: 8895655]
- [10]. Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, Grosschedl R, et al. Functional interaction of beta-catenin with the transcription factor LEF-1. Nature. 1996; 382:638–42. [PubMed: 8757136]

- [11]. Yi XP, Gerdes AM, Li F. Myocyte redistribution of GRK2 and GRK5 in hypertensive, heart-failure-prone rats. *Hypertension*. 2002; 39:1058–63. [PubMed: 12052842]
- [12]. Doglioni C, Piccinin S, Demontis S, Cangi MG, Pecciarini L, Chiarelli C, et al. Alterations of β -Catenin Pathway in Non-Melanoma Skin Tumors: Loss of α -ABC Nuclear Reactivity Correlates with the Presence of β -Catenin Gene Mutation. *The American Journal of Pathology*. 2003; 163:2277–87. [PubMed: 14633602]
- [13]. Oosterwegel M, van de Wetering M, Dooijes D, Klomp L, Winoto A, Georgopoulos K, et al. Cloning of murine TCF-1, a T cell-specific transcription factor interacting with functional motifs in the CD3-epsilon and T cell receptor alpha enhancers. *The Journal of Experimental Medicine*. 1991; 173:1133–42. [PubMed: 1827138]
- [14]. Travis A, Amsterdam A, Belanger C, Grosschedl R. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function [corrected]. *Genes & Development*. 1991; 5:880–94. [PubMed: 1827423]
- [15]. Verbeek S, Izon D, Hofhuis F, Robanus-Maandag E, te Riele H, Watering Mvd, et al. An HMG-box-containing T-cell factor required for thymocyte differentiation. *Nature*. 1995; 374:70–4. [PubMed: 7870176]
- [16]. Castrop J, van Wichen D, Koomans-Bitter M, van de Wetering M, de Weger R, van Dongen J, et al. The human TCF-1 gene encodes a nuclear DNA-binding protein uniquely expressed in normal and neoplastic T-lineage lymphocytes. *Blood*. 1995; 86:3050–9. [PubMed: 7579399]
- [17]. Korinek V, Barker N, Willert K, Molenaar M, Roose J, Wagenaar G, et al. Two Members of the Tcf Family Implicated in Wnt/beta -Catenin Signaling during Embryogenesis in the Mouse. *Mol Cell Biol*. 1998; 18:1248–56. [PubMed: 9488439]
- [18]. Lee Y, Swencki B, Shoichet S, Shivdasani RA. A Possible Role for the High Mobility Group Box Transcription Factor Tcf-4 in Vertebrate Gut Epithelial Cell Differentiation. *Journal of Biological Chemistry*. 1999; 274:1566–72. [PubMed: 9880534]
- [19]. Barker N, Huls G, Korinek V, Clevers H. Restricted High Level Expression of Tcf-4 Protein in Intestinal and Mammary Gland Epithelium. *The American Journal of Pathology*. 1999; 154:29–35. [PubMed: 9916915]
- [20]. Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J, et al. Revision of the 1990 Working Formulation for the Standardization of Nomenclature in the Diagnosis of Heart Rejection. *The Journal of Heart and Lung Transplantation*. 2005; 24:1710–20. [PubMed: 16297770]
- [21]. Tan CD, Baldwin WM, Rodriguez ER. Update on Cardiac Transplantation Pathology. *Archives of Pathology & Laboratory Medicine*. 2007; 131:1169–91. [PubMed: 17683180]
- [22]. Luthringer DJ, Yamashita JT, Czer LS, Trento A, Fishbein MC. Nature and significance of epicardial lymphoid infiltrates in cardiac allografts. *J Heart Lung Transplant*. 1995; 14:537–43. [PubMed: 7654737]

Figure 1.

Morphology, β -catenin and TCF/LEF expression in the epicardium. A to D, H&E morphology. Control native hearts (A) have a thin layer of fibrous tissue on the surface above the underlying fat and show no inflammatory infiltrate. Explanted allografts demonstrate diffuse fibrosis on epicardial surface (B and C upper) and underlying fat (B and C lower) with (B) or without lymphoid aggregates (C). There are also lymphoid aggregates without associated fibrosis (D). E to F, TCF4 immunohistochemistry. Only rare faint TCF4 positive cells are present in the epicardium of native hearts (E) while fibroblasts and lymphocytes (lower left corner) of fibrotic epicardium from explanted allografts show strong positivity for TCF4 (F). G to I, β -catenin immunohistochemistry. There are rare faint β -catenin positive cells in the epicardium of native hearts (G) while fibroblasts (H) and lymphocytes (I) of fibrotic epicardium from explanted allografts are positive for β -catenin (H and I). J and K, TCF1 (J) and LEF1 (K) are only expressed in lymphocytes, but not fibroblasts in the epicardium of explanted allografts. L, negative control with lymphoid aggregate and adjacent fibrosis of explanted allografts. Original magnifications: A to D, $\times 100$; E to L, $\times 400$.