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Galectin-3 is a marker of myocardial and vascular fibrosis in Kawasaki disease patients with giant aneurysms☆

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ABSTRACT

Backgrounds: Galectin-3 (Gal-3) is a multifunctional matricellular protein associated with heart failure and cardiovascular events. Gal-3 is required for transforming growth factor-β pathway-mediated myofibroblast activation that is a key process in coronary artery aneurysm formation in Kawasaki Disease (KD). Autopsies from young adults late after KD onset (AKD) have demonstrated bridging fibrosis throughout the myocardium and arteries. In this study, we postulated that Gal-3 may participate in the pathogenesis of myocardial and vascular fibrosis and the remodeling of coronary artery aneurysms following acute KD.

Methods and results: We measured plasma Gal-3 levels in 63 pediatric KD (PKD) and 81 AKD subjects. AKD subjects with giant aneurysms had significantly higher Gal-3 levels compared to the other adult groups (all $p < 0.05$). All PKD groups had significantly higher Gal-3 levels than pediatric healthy controls (HC) (all $p < 0.05$). Histological and immunohistochemical staining was performed on tissues from 10 KD autopsies and one explanted heart. Gal-3 positive staining was detected associated with acute inflammation and in spindle-shaped cells in the myocardium and arterial wall in KD subjects with giant aneurysms.

Conclusions: AKD subjects with giant aneurysms and PKD subjects had significantly higher plasma Gal-3 levels than HC and Gal-3 expression was increased in the myocardium of KD subjects who died with either acute inflammation or marked myocardial fibrosis. Gal-3 may be a clinically useful biomarker that identifies a subset of KD patients at highest risk of myocardial and vascular fibrosis, and may be an attractive therapeutic target to prevent myocardial dysfunction in this subset.

1. Introduction

Kawasaki disease (KD) is a self-limited, acute vasculitis of young children whose etiology remains unknown. Coronary artery aneurysms (CAA) are the most significant complication, and are found in 15–25% of untreated patients and 3–5% treated with intravenous immunoglobulin (IVIG) [1,2]. Thrombosis of these aneurysms or stenosis due to luminal myofibroblastic proliferation can lead to myocardial infarction, ischemic heart disease, or sudden death. Myocarditis is associated with coronary artery vasculitis in the majority of KD cases based on histologic studies of autopsies and endomyocardial biopsies [3–5]. Emerging recognition of the long-term significance of myocardial fibrosis in young adults following KD is based largely on case reports from cardiac transplantation and small series of autopsy cases [6].

Galectin-3 (Gal-3) is a β-galactoside-binding lectin and a matricellular protein that plays a multifunctional role in inflammation, fibrosis, and cell differentiation [7] and is required for transforming growth factor (TGF)-β pathway-mediated myofibroblast activation [8] that is a key process in CAA formation in KD [9]. Gal-3 is expressed by fibroblasts [10] and inflammatory cells including monocytes/macrophages [11–13]. Gal-3 is recognized as a marker of heart failure and cardiovascular events [14–16], and autopsies in young adults late after KD onset have demonstrated bridging fibrosis throughout the myocardium and arteries [6]. We postulated that Gal-3 may participate in the pathogenesis of myocardial and vascular fibrosis and the remodeling of CAAs following acute KD. To

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test this hypothesis, we measured plasma Gal-3 levels in acute and convalescent KD patient plasma samples and stained for Gal-3 in the myocardium and coronary arterial walls from KD autopsy and cardiac transplant cases.

2. Methods

2.1. Subjects

We enrolled 63 pediatric subjects who met American Heart Association clinical criteria for complete KD [17] at Rady Children's Hospital San Diego (Table 1 and supplemental table A.2) and 81 adult subjects with history of KD who participated in the San Diego Adult KD Collaborative Study. The investigation was performed according to the Declaration of Helsinki, and the Institutional Review Board of the University of California San Diego reviewed and approved this study. All subjects or their parents gave written informed consent for study participation. For the adult KD subjects, giant aneurysm was defined as a CAA with an internal diameter of at least 8 mm as measured by computed tomography (CT) or standard coronary angiography. The pediatric subjects were defined as having normal coronary arteries if the internal diameter of the right coronary artery and left anterior descending coronary artery (RCA and LAD) normalized for body surface area and expressed as standard deviation units from the mean (Z-score) was less than 2.5 as determined by transthoracic echocardiography. Giant aneurysm was defined as a Z-score greater than 10 and an aneurysm was defined as a Z-score greater than 4.0 and less than 10. For comparison, we studied 68 normotensive, age-similar and sex-matched healthy adult control subjects (median age 23.4 years, IQR 21.8–27.0 years; 41% male) who were recruited from our university campus and seven normotensive pediatric healthy controls who were age-similar patients undergoing minor orthopedic surgery procedures.

2.2. Sample collection

Blood samples from pediatric KD patients were collected at the following time points: acute (pre-treatment), early convalescent (illness day 21–82), and late convalescent (illness day 263–4396). Illness Day 1 was considered to be the first day of fever. Clinical laboratory data were also collected for pediatric KD subjects from the same phlebotomy sample used for Gal-3 measurements. Blood was collected in tubes containing sodium EDTA and plasma was separated immediately by centrifugation and stored at −80 °C.

Galectin-3 assay: Gal-3 levels were measured by enzyme-linked immunosorbent assay (ELISA) (BG Medicine, Waltham, MA, USA). The intra-assay and inter-assay coefficients of variation were 0.8% to 6.1% and 1.0% to 4.9%, respectively.

2.3. Tissue samples

We obtained formalin fixed, paraffin-embedded tissues from 11 KD patients (Table 2 and Supplemental Table A.1). Seven of these patients had giant aneurysms (Cases 1–7). The four remaining patients did not have aneurysms but died of other causes after recovery from KD (Cases N-1 to N-4). Tissues were obtained either at the time of autopsy (Cases 1, 2, 4, and 5, Cases N-1–N-4) or surgery (Cases 3, 6, and 7) following written informed consent from the subjects or their parents. The tissue sampling protocol was approved by the Investigational Review Board of the University of California San Diego and Toho University Ohashi Medical Center. The clinical details of the cases are summarized in Table 2 and Supplemental Table A.1. We also obtained control tissue samples from children who died from complications of congenital diaphragmatic hernia and from adults who died of Hodgkin lymphoma. All tissues (5 μm sections) were stained with hematoxylin and eosin (H&E) and Masson's trichrome stain according to standard protocols.

Immunohistochemistry (IHC): All tissues were fixed in formalin and embedded in paraffin. Tissue sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol. Antigen retrieval was performed either in citrate buffer in a microwave oven for 10 min. Slides were incubated with 1.5% normal swine serum blocking solution (Vector Laboratories) at room temperature for 60 min then incubated in PBS with 1.5% goat or horse serum overnight at 4 °C with anti-Galectin-3 antibody (rabbit polyclonal H-160: sc-20157, Santa Cruz Biotechnology). Specificity of this Gal-3 antibody was shown by Western blotting with images available at the company website (http://www.scbt.com/datasheet-20157-galectin-3-h-160-antibody.html). Antibodies were detected using biotin–avidin, LSAB2 System-HRP kit (DAKO K0675), and followed by coloring with AEC peroxidase substrate kit (Vector SK4200) according to the manufacturer's instructions. Negative staining controls included normal rabbit immunoglobulin G (IgG) (Dako, Cat.X0936) as the first antibody. Digital microscopic images were captured by Nanozoomer 2.0HT using NanoZoomer Digital Pathology (NDP) v2.1 (Hamamatsu Photonics K.K., Japan).

Immunofluorescent double-staining for α-SMA and Gal-3 were performed as previously described [9]. Binding of antibodies was detected following incubation with donkey anti-mouse IgG Alexa Fluor 488 (Jackson Immunoresearch laboratory, Cat. 715-545-150) (1:100) for α-SMA and donkey anti-rabbit IgG Alexa Fluor 594 (Jackson Immunoresearch laboratory, Cat. 711-585-152) (1:100) for the Gal-3.

2.4. Statistical methods

Data were analyzed by the Kruskal–Wallis test and Mann–Whitney test using Prism software (Graphpad software, La Jolla, CA, USA), and two-tailed $p < 0.05$ was considered to be statistically significant.

Table 1
Demographic and clinical characteristics of the study population.

Characteristic	Pediatric KD			
	Pediatric healthy control (n = 7)	Normal coronary (n = 42)	Aneurysm (n = 14)	Giant aneurysm (n = 7)
Coronary artery Z score[a]	–	<2.5	10 > Z score ≥ 4.0	≥10
Age, months (median, range)[b]	35.4 (10–69)	34.0 (2.0–130)	24.0 (3.0–85)	5.0 (2.0–49)
% Male	57	53	60	100
Coronary Z score worst (median, IQR)	–	1.2 (0.7–1.7)	4.4 (4.1–5.5)	14.8 (11.7–25.7)
Illness Day at diagnosis, (range)	–	6 (2–14)	6.5 (3–11)	10 (4–19)
Early convalescent time point, (days, range)	–	47 (25–71)	39 (21–55)	37 (28–82)
Late convalescent time point, (years, range)	–	1.36 (0.99–12.04)	1.82 (0.72–11.84)	1.99 (0.94–2.25)

IQR: interquartile range.
[a] Coronary artery Z score: internal diameter normalized for body surface area and expressed as standard deviation units from the mean.
[b] Age at sample collection for pediatric healthy controls, age at onset of KD for pediatric KD subjects.