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Original Paper

Activation of TGF-β1/α-SMA/Col I Profibrotic Pathway in Fibroblasts by Galectin-3 Contributes to Atrial Fibrosis in Experimental Models and Patients

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Key Words
Atrial fibrillation • Fibrosis • Galectin-3 • HL-1 cell • Rapid atrial pacing

Abstract
Background/Aims: This study aimed to evaluate whether galectin-3 (Gal-3) contributes actively to atrial fibrosis both in patients and experimental atrial fibrillation (AF) models. ***Methods:*** Mouse HL-1 cardiomyocytes were subjected to rapid electrical stimulation (RES) to explore Gal-3 expression and secretion levels by western blotting (WB) and enzyme linked immunosorbent assay (ELISA). Neonatal rat cardiac fibroblasts were treated with conditioned culture medium and recombinant human Gal-3 to evaluate the activation of the transforming growth factor (TGF)-β1/α-smooth muscle actin (SMA)/collagen I (Col I) profibrotic pathway (WB) and fibroblast proliferation with a Cell Counting Kit-8 (CCK-8). Furthermore, in the rapid atrial pacing (RAP) rabbit AF model, atrial Gal-3 expression and its effects on the profibrotic pathway were evaluated (WB and Masson's trichrome staining). Moreover, 44 consecutive patients who underwent single mitral valve repair/replacement were included, consisting of 28 patients with persistent AF (PeAF) and 16 with sinus rhythm (SR). Coronary sinus blood was also sampled to test circulating Gal-3 levels (ELISA), and atrial myocardium Gal-3 and its downstream TGF-β1/α-SMA pathway were also measured by WB and immunohistochemical staining. ***Results:*** Gal-3 expression in HL-1 cells and its secretion level in culture medium were greatly increased after 24 h RES. Treatment of neonatal rat cardiac fibroblasts with conditioned media collected from the RES group or recombinant human Gal-3 protein (10 and 30 μg/mL) for 72 h induced the activation of the TGF-β1/α-SMA/Col I profibrotic pathway. RAP increased Gal-3 levels and activated the TGF-β1/α-SMA/Col I pathway in rabbit left atria, while the Gal-3 inhibitor N-acetyllactosamine, injected at 4.5 mg/kg every 3 days, mitigated these adverse changes. Furthermore, Gal-3 levels in coronary sinus blood samples and myocardial Gal-3

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expression levels were higher in the PeAF patients than in the SR patients, and higher level profibrotic pathway activation was also confirmed. ***Conclusions:*** Activation of Gal-3 expression in the atria can subsequently activate the TGF-β1/α-SMA/Col I pathway in cardiac fibroblasts, which may enhance atrial fibrosis.

Introduction

Atrial fibrillation (AF) is one of the most common arrhythmias and is associated with an approximately 2-fold increase in mortality compared with sinus rhythm (SR) control populations [1]. Atrial fibrosis is regarded as a hallmark of AF-related structural remodeling and also contributes to the perpetuation of AF [2, 3]. Thus, understanding the process and regulation of atrial fibrosis can help to elucidate the intrinsic mechanism underlying the initiation and maintenance of AF and may have potential clinical therapeutic implications.

The concept that the β-galactoside-binding lectin galectin-3 (Gal-3) is a pro-fibrotic protein has been postulated and studied mostly in the setting of heart failure (HF) [4-8]. More importantly, Gal-3 has been recognized as a novel biomarker for HF by the US Food and Drug Administration. Yu et al [9]., using a rodent animal model, showed that both genetic disruption and pharmacological inhibition of Gal-3 could attenuate cardiac fibrosis and HF development, indicating that Gal-3 contributes actively to HF development instead of just being a bystander. Gurses et al [10]. found that serum Gal-3 levels were higher in paroxysmal AF patients with preserved left ventricular function than in SR patients, while intracardiac serum Gal-3 levels were elevated in persistent AF (PeAF) patients compared with those in paroxysmal AF patients. Moreover, the level of serum Gal-3 has been shown to be significantly associated with atrial remodeling in paroxysmal AF patients with preserved left ventricular function [11], and its level could predict AF recurrence after ablation in PeAF patients without structural heart disease [12] and could also act as a marker of thrombogenicity in AF patients [13]. However, the level of circulating Gal-3 is not specifically related to heart conditions and may be influenced by other factors, such as pulmonary, liver, or kidney fibrosis. Hence, the further evaluation of intra-cardiac coronary sinus serum Gal-3 levels [14, 15] and the direct detection of Gal-3 levels in human atrial myocardium tissues are of great value [16]. Until now, the mechanism by which Gal-3 has an effect on atrial fibrosis in experimental AF models and human patients is far from clear, and its role in AF maintenance, whether as simply a bystander or an active player, remains to be elucidated. In the present study, we aimed to determine whether rapid electrical stimulation (RES), just like the fast heart rhythm in AF patients, could activate Gal-3 expression and secretion in HL-1 cardiomyocyte cells, and we also examined the profibrotic role of Gal-3 in fibroblast cells and a rabbit AF model. Furthermore, we compared Gal-3 levels in coronary sinus blood samples and left atrial myocardium specimens from SR and PeAF patients to explore further the role of Gal-3 in atrial fibrosis.

Materials and Methods

HL-1 cell line culture and RES

Mouse HL-1 cardiomyocyte cells were kindly provided by Dr. William C. Claycomb (Louisiana State University, New Orleans, LA, USA), and the cells were cultured according to his instructions. HL-1 cardiomyocytes were RES treated at 10V/cm in 5Hz frequency which was commonly used as an *in vitro* AF model.

The cells were changed to a serum-free medium (SFM) at 12 h before RES and then cultured with fresh SFM and subjected to RES with a C-Pace EP Culture Stimulator and C-Dish Electrode (IonOptix, Westwood, MA, USA) at a 5-ms duration and 5-Hz square-wave pulses (10 V/cm) for 12 or 24 h, and conditioned media and cell proteins were collected for further analysis. Control cells were cultured in parallel conditions without RES.

Enzyme-linked immunosorbent assay

Gal-3 levels were assayed by commercially available enzyme linked immunosorbent assay (ELISA) kits. The kit for HL-1 cell culture supernatant Gal-3 (F00945) was purchased from Westang Bio-Tech Company (Shanghai, China), while the kit for human serum samples (EK11262) was from Multisciences Bio-Tech Company (Shanghai, China), and the ELISA tests were processed according to the manufacturers' instructions. A standard concentration-absorbency curve was generated for each batch of samples and all samples were assayed in duplicate. Gal-3 concentrations were calculated with a standard curve ranging from 0 to 2000 pg/mL using the optical density detected by a Microplate Reader (Infinite M200; TECAN, Männedorf, Switzerland). The lower limit of detection was less than 16 pg/mL, and the inter- and intra-assay coefficients of variation were <10%.

Neonatal rat cardiac fibroblast isolation, culture, and treatment

Fibroblasts are the main cell type producing the majority of extracellular matrix molecules (collagen, etc.). Cardiac fibroblasts were harvested from neonatal Sprague-Dawley rats younger than 3 days (SLAC Laboratory Animal Co., Shanghai, China), and the fibroblasts were cultured as described previously [17] with minor modifications. Briefly, after the rats were anesthetized and sacrificed by immersion into 75% (v/v) alcohol, the whole heart was dissected and washed 3 times in dissociation buffer (in mmol/L: 116 NaCl, 0.8 Na_2HPO_4, 5.4 KCl, 0.8 $MgSO_4$, 20 HEPES, 5.6 glucose, pH 7.35) and then digested in 0.1% trypsin (Invitrogen, Grand Island, NY, USA) and 0.05% collagenase type II (Invitrogen) at 37 °C. The cell suspension was filtered with a 100-μm nylon cell strainer (Corning Incorporated, Tewksbury, MA, USA) and centrifuged at 1500 × *g* for 10 min. The pellet was resuspended in Dulbecco's modified Eagle's medium containing 20% fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin, and the cells were seeded at 37 °C for a 1-h incubation. The unattached cells were discarded and the attached cells were maintained as cardiac fibroblast cells. Fibroblasts in passages 3–6 were used in all experiments, and at 12 h before each treatment, the fibroblasts were cultured with SFM and then administered 1, 10, or 30 μg/mL Gal-3 or conditioned media from HL-1 cells for 72 h. The cells were collected to detect the expression of fibrosis-related targets.

Cardiac fibroblast proliferation

Cell viability was measured with a Cell Counting Kit-8 (CCK-8) (CK04; Dojindo Molecular Technologies, Kumamoto, Japan). Cardiac fibroblasts were cultured in SFM with recombinant human Gal-3 protein (control, 1 μg/mL, 10 μg/mL, or 30 μg/mL) in a 96-well plate for 24 h. Then, 10 μL CCK-8 solution was added to each well and incubated at 37 °C for 2 h in a 5% CO_2 incubator. Absorbance was measured at a wavelength of 450 nm with a microplate reader (Infinite M200; TECAN, Männedorf, Switzerland) and presented as viability = (absorbance of the samples)/(absorbance of the control).

Establishment of the rapid atrial pacing (RAP) rabbit model

All of the rabbits were anesthetized with a 15 mg/kg (ketamine):5 mg/kg (xylazine) intramuscular injection. The animals were then intubated and mechanically ventilated with a volume-cycled ventilator (Model HX-200; TAIMENG, Chengdu, China). The heart was exposed via the third intercostal space and a custom-designed electrode collar for pacing was sutured to the epicardial surface of the left atrium. The distal end of the electrode lead was tunneled subcutaneously and connected to a pacemaker (programmed for RAP at 1000 bpm, an output of 6 V with 1.0-ms pulse duration; Harbin University of Science and Technology, Harbin, China) in a subcutaneous abdominal pocket. The rabbits in the sham group underwent an identical surgical procedure without RAP. Postoperative care included the administration of analgesics for 3 days and penicillin injection at 30, 000 IU/kg once every 24 h for 7 days. Thereafter, the pacemakers were switched and examined once a week to ensure continuous RAP.

Rabbit AF model studies and experimental methods

Fifteen New Zealand rabbits (male, 2.0–2.5 kg) were obtained from the Animal Facility Center of the Second Military Medical University. Power analysis was performed using GPower 3.1.9.2 software (Faul, Erdfelder, Lang, & Buchner, Germany, 2007), and a sample size of 15 was required for a fixed effect one-way analysis of variance (ANOVA) test with a power of 0.95 and α error of 0.05. In this part of our study, the effect size was approximately 1.6. All study protocols complied with the Guide for the Care and Use of Laboratory Animals (8th edition; National Institutes of Health, Bethesda, MA, USA). A rabbit AF model

was established by rapid atrial pacing (RAP). The rabbits were randomly assigned into 3 groups: sham operation without RAP stimulation (sham group), RAP treatment for 4 weeks (RAP 4w group), and RAP 4-week treatment along with the Gal-3 inhibitor N-acetyllactosamine (N-Lac) (OA08244; Carbosynth China Ltd., Soochow, China) via auricular vein injection at 4.5 mg/kg every 3 days after the initiation of the atrial pacing procedure (RAP+N-Lac group).

At the end of the experiments, the rabbits were sacrificed and the left atria were harvested quickly and divided into small volume aliquots. One piece was fixed in 4% paraformaldehyde (PFA) for Masson's trichrome staining, and the remaining parts were frozen instantly in liquid nitrogen and stored at -80 °C.

Patient inclusion and left atrial myocardium specimen harvesting

Informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in its *a priori* approval by the Ethics Committee of Changzheng Hospital. All consecutive patients who underwent on-pump pure mitral valve replacement/repair in the Department of Cardiothoracic Surgery of Changzheng Hospital from February to August 2016 were screened for our trial; 28 patients, with a clear medical record of AF and reconfirmed by electrocardiography when admitted, were allocated to the PeAF group, and the other 16 patients, who remained SR before surgery, were assigned to the SR control group.

Left atrial (LA) myocardium specimens were obtained from the incision site of the interatrial groove prior to cardioplegia and cardiopulmonary bypass in a non-traumatic way. No complications occurred after tissue sampling. Atrial sample aliquots were put immediately into liquid nitrogen for western blotting or fixed in 4% PFA for 24 h followed by equilibration, OCT compound (4583; Sakura Finetek, Torrance, CA, USA) embedding, and cryosectioning (Cryotome E; Thermo Scientific, Waltham, MA, USA) for immunohistochemical staining.

Human blood sample analysis

Intra-cardiac coronary sinus blood samples were aspirated via the coronary sinus after an incision was made into the left atrium under satisfactory vena cava drainage. Blood was aspirated to sterile pro-coagulation tubes and allowed to clot for 30–60 min. The tubes were then centrifuged for 15 min at 1600 × *g* at 4 °C. Aliquots of the separated serum were then stored at -80 °C until analysis.

Masson's trichrome staining for collagen and immunohistochemistry

Rabbit left atrial samples were fixed, paraffin embedded, and cut into 5-μm sections (RM2255; Leica, Buffalo Grove, IL, USA), and a Masson's trichrome staining kit (60532ES58; Shanghai Yeasen Bio-tech Company, Shanghai, China) was used to evaluate the degree of interstitial fibrosis. The collagen fibers were stained blue, while the myocardium was marked red. The amount of fibrosis in atrial tissues, which was calculated as the collagen/myocardium ratio in each group, was quantitatively analyzed with the software ImageJ version 1.51 (NIH).

Immunostaining was carried out with the primary antibodies specified above, directed against Gal-3 (dilution 1:50, ab2785; Abcam), TGF-B1(dilution 1:50, ab9758 for rabbit samples, ab92486 for human samples; Abcam), and α-SMA(dilution 1:50, ab7817; Abcam) following standard protocols. Specificity was confirmed by changing the primary antibody to a phosphate-buffered saline solution.

Western blot analysis

Cell samples (HL-1 cells and fibroblasts) were suspended (human or rabbit atrial myocardium tissues were homogenized) and sonicated in lysis buffer (in mmol/L: 50 Tris-HCl, 150 NaCl, 10 NaF 10, 1 Na_3VO_4, 5 EGTA, 5 EDTA, 0.5% Triton X-100, 0.5% Na deoxycholate, and 0.1% SDS) with protease inhibitors (P8340; Sigma-Aldrich (Shanghai) Trading Co., Ltd, Shanghai, China) added immediately before use, followed by centrifugation at 13, 000 × *g* at 4 °C for 15 min. The supernatants were used as whole cell proteins for western blotting.

The protein samples were subjected to one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 8–15% Bis-Tris gels. Proteins were transferred to a polyvinylidene difluoride membrane and the following primary antibodies were used to probe specific proteins independently: anti-Gal-3 (1:1000 dilution, ab2785; Abcam, Cambridge, MA, USA), anti-Col-I (1:1000 dilution, ab6308; Abcam), anti-α-SMA(1:1000 dilution, ab7817; Abcam), anti-TGF-B1(1:1000 dilution, ab9758 for rabbit samples, ab92486

for HL-1 cell samples; Abcam), and anti-GAPDH (internal control, 1:10000 dilution, ab8245; Abcam). After being washed with a phosphate-buffered saline with 0.1% Tween 20 solution 3 times, horseradish peroxidase-conjugated anti-mouse (1:5000 dilution, ab6789; Abcam) or anti-rabbit (1:5000 dilution, ab6721; Abcam) IgGs were used as secondary antibodies. The immunoreactions were visualized using a SuperSignal™ West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Waltham, MA, USA) under a ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, Hercules, CA, USA) and the protein bands were quantified with the software ImageJ version 1.51 (National Institutes of Health [NIH], Bethesda, MA, USA).

Statistical analysis

Continuous variables are expressed as the mean ± standard error of the mean or as number and percentage for dichotomous variables. Continuous variables were tested for normality using the Kolmogorov-Smirnov test and then compared between groups with the unpaired Student's *t*-test if normally distributed or the Mann-Whitney *U* test if not normally distributed. In the case of dichotomous or categorical variables, Pearson's chi-square or Fisher's exact tests were used as appropriate. Multiple group comparisons were measured by ANOVA with Bonferroni's post hoc test. Statistical analysis was performed using SPSS V20.0 software (IBM-SPSS, Chicago, IL, USA). $P < 0.05$ was considered to indicate statistical significance.

Results

RES induces Gal-3 expression and secretion in a time-dependent manner

As shown in Fig. 1, RES activated Gal-3 expression in HL-1 cells in a time-dependent manner; both the 12 and 24 h groups showed a great increase compared with the control group (Fig. 1A, 1B). In addition, we also detected the levels of Gal-3 secretion in culture media at the indicated times by enzyme linked immunosorbent assay. Compared with the control group, the 24 h group exhibited a significant increase of Gal-3 secretion into the culture media (Fig. 1C).

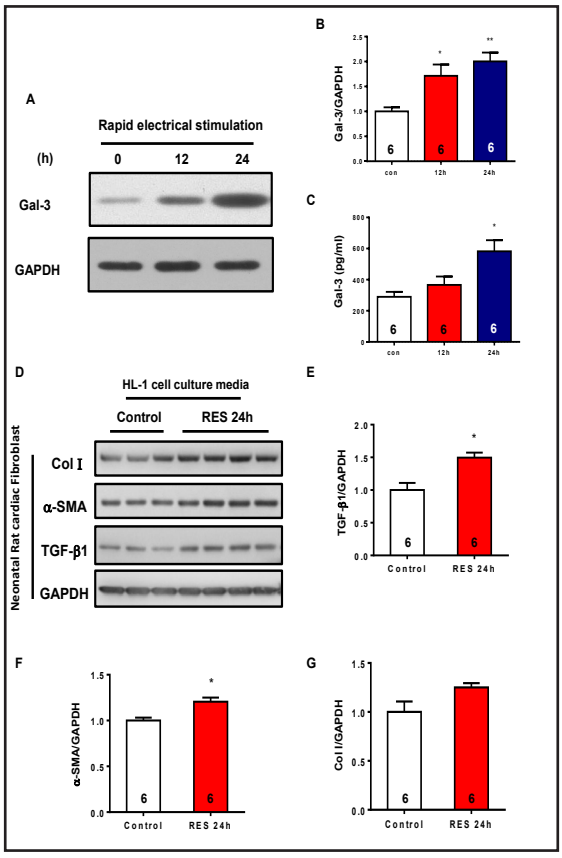

Fig. 1. Rapid electrical stimulation (RES) induces galectin-3 (Gal-3) expression and secretion in HL-1 cells and Gal-3-enriched conditioned media activate transforming growth factor (TGF)-β1/α-smooth muscle actin (SMA)/collagen I (Col I) expression in neonatal rat fibroblast cells. A–B. Representative western blot (A) and summary data (B). HL-1 cells were treated with RES for the indicated times, and Gal-3 protein expression was examined by western blot analysis. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (Compared with control, *P<0.05, **P<0.01, n = 6.) C. HL-1 cells were treated with RES for the indicated times, and Gal-3 secretion levels in culture media were examined by enzyme linked immunosorbent assay. (Compared with control, *P<0.05, n = 6.) D. Representative western blot. Neonatal rat cardiac fibroblast cells were treated with conditioned HL-1 culture media for 72 h, and TGF-β1/α-SMA/Col I expression levels were examined by western blot analysis. E–G. Summary data. Neonatal rat fibroblast cells were cultured either in control serum-free medium (SFM) or conditioned media for 72 h. TGF-β1/α-SMA (F), and Col I (G) expression levels were detected with western blotting. Data were normalized to GAPDH. (Compared with control, *P<0.05, **P<0.01, n = 6.)

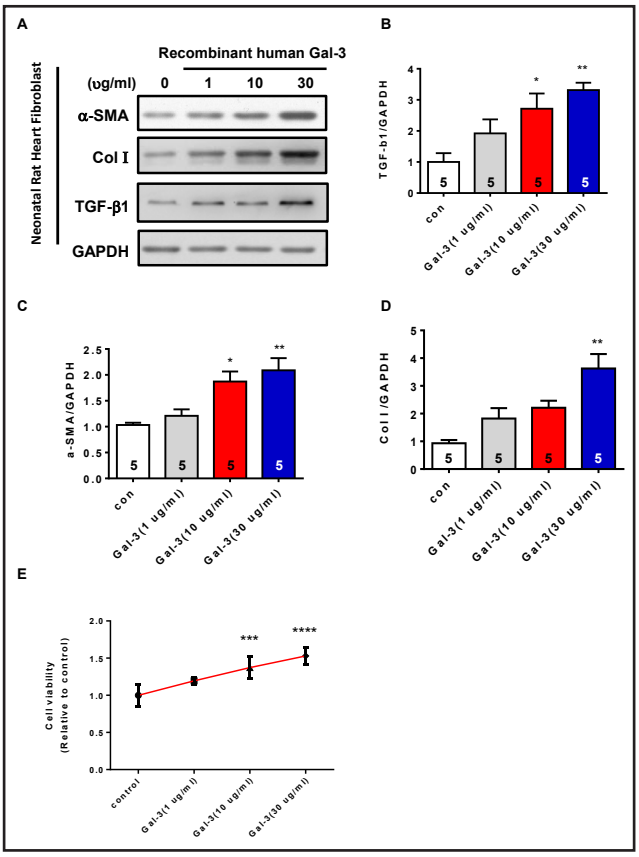

Fig. 2. Recombinant human Gal-3 protein activates the TGF-β1/α-SMA/Col I profibrotic pathway and enhances the proliferation of neonatal rat cardiac fibroblast cells. A–D. Representative western blot (A) and summary data (B–D) of the effects of different concentrations (1, 10, and 30 μg/mL) of recombinant human Gal-3 protein on the profibrotic pathway after 72 h. Data were normalized to GAPDH and quantitated relative to serum-free medium (SFM) control levels. E. Effect of different concentrations (1, 10, and 30 μg/mL) of recombinant human Gal-3 protein on the proliferation of neonatal rat fibroblast cells. (Compared with control, *P<0.05, **P<0.01, n = 5.)

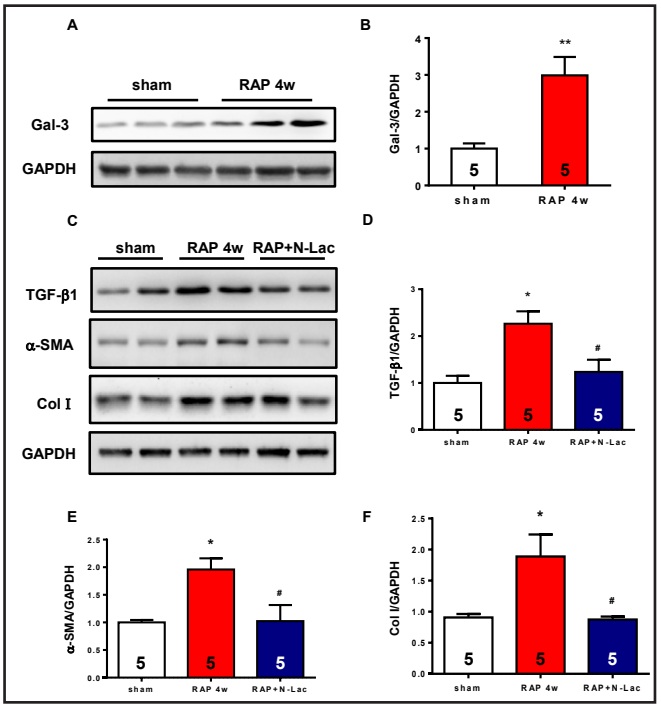

Fig. 3. Rapid atrial pacing (RAP) in rabbits induces Gal-3 expression and its role in the activation of the TGF-β1/α-SMA/Col I pathway and fibrosis in atrial tissues. A–B. Representative western blot (A) and summary data (B) of Gal-3 protein levels after RAP for 4 weeks (4w). Data were normalized to GAPDH and quantitated relative to sham levels. C. RAP increases the levels of TGF-β1/α-SMA/Col I in rabbit atrial tissues; the effects are blocked by the administration of the Gal-3 inhibitor N-acetyllactosamine (N-Lac) at a final dose of 4.5 mg/kg every 3 days from the onset of RAP. Summary data of TGF-β1 (D), α-SMA (E), and Col I (F) expression levels were detected with western blotting. Data were normalized to GAPDH. (Compared with control, *P<0.05, **P<0.01; compared with the RAP group, #P<0.05, n = 5).

Effect of conditioned culture media from HL-1 cells on neonatal rat cardiac fibroblasts

We cultured fibroblasts in conditioned media collected from HL-1 cells that secreted increased levels of Gal-3 to determine whether the conditioned media would lead to an increase

in collagen secretion by fibroblasts. As shown in Fig. 1, compared with control media, RES 24 h conditioned media activated transforming growth factor (TGF)-β1/α-smooth muscle actin (SMA)/collagen I (Col I) expression in fibroblasts after 72 h culture; both TGF-β1/α-SMAshowed a statistically significant increase (Fig. 1E, 1F), while Col I showed a trend for upregulation, although it did not reach statistical significance (Fig. 1G).

Effect of human recombinant Gal-3 on neonatal rat cardiac fibroblasts

To determine whether the activation of the profibrotic pathway was due to the elevated secretion of Gal-3, we further treated the fibroblasts with recombinant human Gal-3 at various concentrations (1, 10, or 30 μg/mL) for 72 h to examine TGF-β1/α-SMA/Col I activation in neonatal rat cardiac fibroblasts. As shown in Fig. 2A–2D, treatment with human recombinant Gal-3 for 72 h induced a significant

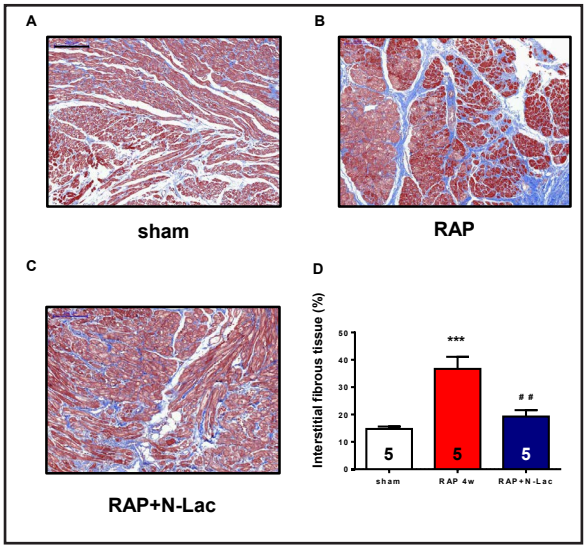

Fig. 4. Gal-3 inhibitor blocks the atrial fibrosis induced by RAP treatment in rabbits. A–C. Representative Masson's trichrome staining of left atrial tissues from rabbits. D. Percentage of Masson's trichrome blue staining area showing interstitial fibrosis from sham (n = 5), RAP treated for 4 weeks (n = 5), and RAP with N-Lac injection (n = 5) groups. (Compared with the sham group, ***P<0.001; compared with the RAP group, ##P<0.01. Scale bar = 200 μm.)

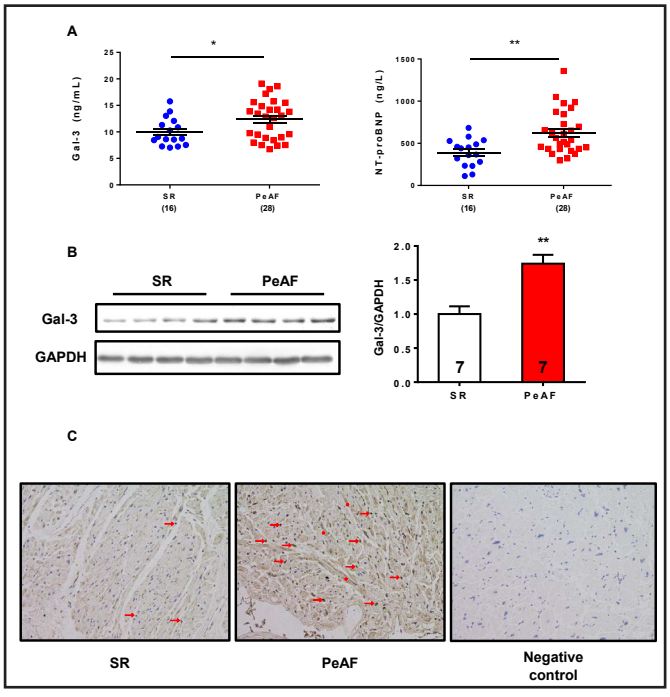

Fig. 5. Gal-3 levels in blood samples and left atrial (LA) tissues from cardiac surgery patients. A. Comparison of coronary sinus serum Gal-3 levels in the sinus rhythm (SR) group and persistent atrial fibrillation (PeAF) patients (left), and comparison of coronary sinus NT-proBNP levels in the SR group and PeAF patients (right). "●" Indicates SR patients and "■" indicates PeAF patients. (Compared with SR, *P<0.05, n = 16 for SR, n = 28 for PeAF.) B. Gal-3 expression levels in human LA myocardium specimens. Representative western blot (left) and summary data (right) of Gal-3 protein levels in LA myocardium specimens from SR and PeAF patients. Data were normalized to GAPDH and quantitated relative to the levels in the SR patient group. (Compared with SR, **P<0.01, n = 7 for SR, n = 7 for PeAF.) C. Immunohistochemical localization of Gal-3 in human left atrial myocardium samples. In LA samples from SR patients (left), some round cells (macrophage cells) were positive for Gal-3 (red arrows), while in the PeAF LA samples, considerably more round cells were positive for Gal-3 (red arrows), and even some cardiomyocytes (red asterisks) showed positive staining. No specific reaction product was seen in the negative controls incubated with non-immune sera.

activation of the TGF-β1/α-SMA/Col I profibrotic pathway at Gal-3 concentrations as low as 10 μg/mL, while Col I showed a marked increase only with 30 μg/mL treatment.

Moreover, to determine the influence of Gal-3 on the proliferation of primary cardiac fibroblasts, we seeded fibroblasts in a 96-well plate and added the same concentrations of human recombinant Gal-3 and detected proliferation using CCK-8. Compared with the serum-free group (control group), 10 and 30 μg/mL Gal-3 both markedly increased the viability of fibroblasts at 72 h, while 1 μg/mL Gal-3 had no effect on proliferation (Fig. 2E).

RAP activates Gal-3 expression and enhances fibrosis in rabbit atrial tissue

To explore further the role of Gal-3 in atrial fibrosis, we established a RAP rabbit model to simulate the pathophysiological processes of PeAF. RAP for 4 weeks at 1000 bpm caused a remarkable increase in Gal-3 levels in atrial tissues compared with the sham group (Fig. 3A–3B), as well as an increase of the protein levels of TGF-β1/α-SMA/Col I (Fig. 3C–3F), while there was no significant difference in Gal-3 levels in left ventricular tissues between both groups (data not shown). Surprisingly, administration of the Gal-3 inhibitor N-Lac at 4.5 mg/kg every 3 days at the onset of cardiac pacing attenuated the activation of the profibrotic pathway in the atria, and more importantly, mitigated the excessive interstitial fibrosis in left atria induced by RAP (Fig. 4).

Table 1. Baseline demographic, clinical, laboratory, medication, and echocardiographic parameters of the study population (n = 44). Values are mean ± standard deviation or n (%). ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; BMI, body mass index; CS, coronary sinus; LAD, left atrial diameter; LVDd, left ventricular end diastolic diameter; LVDs, left ventricular end systolic diameter; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; PAP, pulmonary arterial pressure; PeAF, persistent atrial fibrillation; PV, peripheral vein; SR, sinus rhythm

Baseline characteristics	SR (n=16)	PeAF (n=28)	P value
Clinical characteristics			
Age(years)	57.3 ± 9.4	63.9 ± 7.4	0.014
Male gender	7 (43.8%)	11 (39.3%)	1.000
BMI（kg/m²)	21.7 ± 3.3	23.8 ± 3.1	0.045
NYHA functional class			
II	9	9	0.202
III	7	19	
Co-mobidities			
Coronary heart disease	4 (25%)	19 (67.9%)	0.011
Hypertension	5 (31.3%)	16 (57.1%)	0.125
Diabetes mellitus	4 (25%)	15 (53.6%)	0.113
Laboratory tests			
CS Galectin-3 (ng/ml)	9.98 ± 2.6	12.36 ± 3.7	0.030
PV Galectin-3 (ng/ml)	11.25 ± 2.8	13.39 ± 3.5	0.043
Triglyceride (mmol/L)	1.06 ± 0.4	1.08 ± 0.3	0.830
Cholesterol (mmol/L)	3.13 ± 0.9	3.51 ± 1	0.237
Creatinine (umol/L)	64.87 ± 12.8	66.51 ± 12.9	0.685
NT-proBNP (ng/L)	387.7 ± 162.2	627.6 ± 257	0.002
Medication			
Diuretics	5 (31.3%)	18 (64.3%)	0.0592
β-Blockers	5 (31.25%)	20 (71.4%)	0.013
Digitalis	6 (37.5%)	14 (50%)	0.534
Calcium antagonist	5 (31.3%)	15 (53.6%)	0.213
ACE inhibitors/ARBs	6 (37.5%)	19 (67.9%)	0.065
Aldosterone antagonist	6 (37.5%)	14 (50%)	0.534
Statins	4 (25%)	18 (64.3%)	0.027
Nitrate	6 (37.5%)	15 (53.6%)	0.360
Echocardiography			
LAD (mm)	41.9± 5.2	48 ± 6	<0.01
LVDd (mm)	50.4 ± 2.9	52.6 ± 1.6	0.456
LVDs (mm)	33.4 ± 2	36.2 ± 1.2	0.207
LVEF (%)	57 ± 6.2	52.6 ± 8.3	0.110
PAP (mmHg)	39.6 ± 1.8	41.1 ± 1.1	0.435

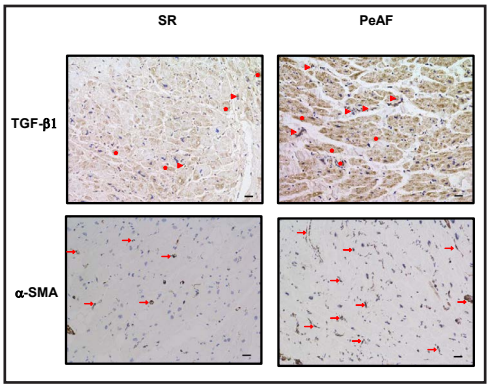

Fig. 6. Activation of TGF-β1/α-SMA in LA samples from PeAF patients compared to those from SR patients. For TGF-β1 (upper), some cardiomyocytes (red asterisks) and fibroblasts/myofibroblasts (triangles) were positive for TGF-β1 in the SR patients, but more TGF-β1-positive cells were observed in the PeAF samples. For α-SMA (lower), α-SMA-positive cells were marked with a red arrow in the SR patients, and more α-SMA-positive cells were observed in the PeAF samples compared to the SR samples. (n = 7 for SR, n = 7 for PeAF. Original magnification, 200 ×; scale bar = 100 μm.)

Gal-3 levels in coronary sinus serum samples of cardiac surgery patients

We included 44 patients who underwent on-pump pure mitral valve replacement/repair surgery (Table 1), consisting of 28 patients with PeAF and 16 patients with SR. Gal-3 levels were measured in blood samples from the coronary sinus after an interatrial groove incision was made under satisfactory vena cava drainage. The results showed that intracardiac serum Gal-3 levels were higher in the PeAF patients than in the SR patients (Fig. 5A, left). Besides, N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations were also greatly elevated in the PeAF patients compared with those in the SR patients (Fig. 5A, right).

Gal-3 expression and fibrotic pathway activation in human LA samples

In addition to its circulating levels, we also measured the expression levels of Gal-3 in LA myocardium specimens harvested during surgery. Western blotting showed that Gal-3 levels were significantly higher in atrial tissues from the PeAF patients than in those from the SR patients (Fig. 5B), which was also confirmed by immunohistochemistry (Fig. 5C). Furthermore, we evaluated the expression of TGF-β/α-SMAin human LA samples (Fig. 6). We detected the increased expression of TGF-β1/α-SMAin the PeAF patients compared with the SR patients, which may underlie the enhanced atrial fibrosis found in the former subjects.

Discussion

The mechanisms of AF are nowadays recognized to be associated with structural factors, electrical remodeling, and calcium signaling abnormalities in the atria [18]. Targeting fibrosis is regarded as a potential strategy for the treatment of AF [19], while inhibition of Gal-3 has been shown to attenuate cardiac fibrosis and remodeling and prevent the development of HF in animal studies [9]. Thus, we hypothesized a possible link between Gal-3 and interstitial fibrosis in the atria during AF.

This study demonstrates several findings that Gal-3 is not simply a bystander but an active contributor to atrial fibrosis. Gal-3 is expressed predominantly by fibroblasts and macrophages in the majority of previous studies [20-22]. However, some also reported that Gal-3 is expressed in ventricular myocytes in a pressure-overloaded remodeling heart model and that a high density of Gal-3-positive myocytes is related to abnormalities in remodeling and heart function [23]. Furthermore, HL-1 cells have been demonstrated to express Gal-3 at baseline and remarkably induce its expression following protein kinase C activation, indicating that cardiomyocytes may also act as a source of Gal-3 in the heart [24] and possibly be greatly induced in pathological conditions. Moreover, it has long been verified that rapidly-paced cardiomyocytes release substances that profoundly alter cardiac fibroblast function, although without identification of candidate molecules contributing to this effect [25]. Here, we elucidated that RES, a widely used method to establish an *in vitro* AF model, could significantly induce Gal-3 expression and secretion in HL-1 cells. This finding suggests that HL-1 cells may synthesize Gal-3 endogenously and influence nearby myocytes or fibroblasts via Gal-3 secretion. As Gal-3 has been shown to be closely involved in many fibrotic diseases [26], we focused on the effect of Gal-3 on the typical profibrotic pathway in the atria.

Among the complicated fibrosis signaling networks, the TGF-β1/α-SMA/Col I profibrotic pathway has been widely recognized to induce cardiac hypertrophy and fibrosis in the failing heart [27]. Briefly, in the typical TGF- β1 pathway [28], TGF-β1 binds to the receptors on fibroblasts and myocytes, thereby activating the Smad 2/3/4 complex to facilitate its nuclear translocation and initiating the synthesis and secretion of pro-fibrotic cytokines, such as TGF-β1, procollagen, and α-SMA, which could induce fibroblasts to differentiate into myofibroblasts [29]. The enhanced presence of cardiac fibroblast and myofibroblast differentiation in the atrial interstitial space has been reported in various AF fibrosis substrates [2]. A previous study reported that 10 μg/mL Gal-3 increased the proliferation

of sheep atrial myofibroblasts, while this effect could be inhibited by a Gal-3 inhibitor [30]. Importantly, Gal-3 is known to retain TGF-β receptors on the myofibroblast cell membrane, thereby promoting profibrotic signaling through the Smad and Akt pathways and stimulating matrix production [31]. Consistent with this result, we found that the TGF-β1/α-SMA/Col I pathway was induced in neonatal rat cardiac fibroblasts by Gal-3-enriched conditioned HL-1 culture media, and further confirmed the role of Gal-3 in fibroblast differentiation and proliferation with recombinant human Gal-3 protein treatment, both at 10 and 30 μg/mL; exogenous Gal-3 activated the profibrotic pathway and induced fibroblast proliferation, as detected by CCK-8. All of these results led us to the conclusion that although cardiomyocytes barely express Gal-3 under physiological conditions [9], under pathological conditions, cardiomyocytes may also act as an alternative source of Gal-3 [23, 24], inducing fibroblasts to synthesize collagen and other pro-fibrotic components of the extracellular matrix.

As the profibrotic effects of Gal-3 were explored *in vitro*, we further tried to demonstrate that *in vivo* inhibition of Gal-3, regardless of whether it originates from macrophages, fibroblasts, or even cardiomyocytes, could help to inhibit the fibrotic process in the RAP rabbit model. RAP has been used commonly to induce AF in sheep [32], canines [33], and rabbits [34]. Studies have shown that RAP on the left atrium of rabbit heart at 1000 ppm for 4 weeks caused significant atrial fibrosis [34], and in our study, the TGF-β1/α-SMA/Col I pathway was remarkably activated in the atria of the RAP alone group, along with the significant deposition of collagen, as estimated by Masson's trichrome staining, indicating that tachycardia during AF may cause atrial structural remodeling, such as fibrosis, which promotes the perpetuation of AF. Furthermore, we explored the role of Gal-3 in this pro-fibrotic process, and the results indicated that Gal-3 was greatly induced by RAP and treatment with the Gal-3 inhibitor N-Lac during RAP; TGF-β1/α-SMA/Col I were normalized to levels similar to those of the sham group. In addition, protection against interstitial fibrosis was further confirmed by a significant decrease in rabbit atrial fibrosis.

Next, we endeavored to extrapolate our findings to clinical patients. A clear correlation between Gal-3 serum levels and interstitial fibrosis in clinical LA samples has been reported, although no causality was implied [16]. Elevated serum Gal-3 levels in AF patients have also been suggested to be driven by cardiometabolic comorbidities, but not heart rhythm [35]. Therefore, in this part of the study, we tried to understand the role of serum Gal-3 in AF patients after establishing its effects in cellular and animal models. A recent clinical trial suggested that coronary sinus sampling of HF biomarkers, such as Gal-3, may be more appropriate than peripheral venous blood levels for predicting the outcome of cardiac resynchronization therapy [15]. Thus, we compared the levels of coronary sinus serum Gal-3 in patients who underwent pure mitral valve cardiac surgery. Our data showed that PeAF patients had higher levels of circulating Gal-3 than SR control patients. This is in agreement with another study showing that persistent atrial fibrillation was an independent predictor of higher Gal-3 concentrations [11], and Gal-3 levels were found to be higher in patients with persistent atrial fibrillation and in those with cardiovascular risk factors [35]. With respect to sampling site differences, circulating Gal-3 in AF patients possibly originates not only from the heart but could also reflect systemic fibrosis and inflammation levels, and as reported previously, Gal-3 levels are higher peripherally than in intra-cardiac samples from patients undergoing catheter ablation for atrial fibrillation [30]. This is the reason why we chose the coronary sinus as the sampling site to exclude interference from systemic fibrosis and inflammation. All of the above results indicate that serum Gal-3 levels were higher in persistent AF patients than in SR controls, and intracardiac Gal-3 can be used as a potential fibrosis biomarker in atrial samples. Besides, plasma NT-proBNP levels were significantly higher in patients with paroxysmal and persistent AF compared to those with SR in the setting of preserved left ventricular systolic function [36]; thus, we also tested serum NT-proBNP levels, and the results also validated our previous conclusions that PeAF patients have higher coronary sinus serum NT-proBNP levels than SR controls.

As an emerging biomarker in heart fibrosis, the expression levels of Gal-3 in human atrial tissues are limited, although its circulating levels have been studied in various trials

[37]. We confirmed the elevated expression of Gal-3 in LA samples from PeAF patients by both western blotting and immunohistochemistry methods, further establishing a role for Gal-3 in human atrial fibrosis. Moreover, the downstream effectors of the pro-fibrotic signal pathway were also confirmed by showing that TGF-β1/α-SMAwere both markedly activated in LA samples of PeAF patients. As the overexpression of cardiac TGF-B1has been shown to promote atrial fibrosis, conduction abnormalities, and AF in a mouse model [38], and myofibroblasts (α-SMA-positive cells) are key mesenchymal cells implicated in extracellular matrix synthesis [39], the activation of the Gal-3/TGF-β1/α-SMAprofibrotic pathway could have an active role in extracellular matrix synthesis and interstitial fibrosis in AF patients.

Conclusion

RES of HL-1 cells induced Gal-3 expression and secretion in a time-dependent manner *in vitro*. Both Gal-3-enriched conditioned HL-1 culture media and recombinant human Gal-3 protein activated the TGF-β1/α-SMA/Col I pathway in neonatal rat fibroblast cells, and fibroblast proliferation was also boosted by recombinant human Gal-3 protein. *In vivo*, RAP in a rabbit model activated the Gal-3/TGF-β1/α-SMA/Col I profibrotic process and resulted in the accumulation of interstitial fibrosis in left atria, while these adverse effects were attenuated by the administration of the Gal-3 inhibitor N-Lac. Furthermore, coronary sinus serum Gal-3 levels were higher in PeAF patients than in SR patients, and Gal-3/TGF-β1/α-SMApathway activation was also confirmed in LA specimens from patients. Taken together, these findings show that Gal-3 contributes actively to atrial fibrosis via activation of the TGF-β1/α-SMA/Col I profibrotic pathway in an AF experimental model and patients. Thus, we propose that Gal-3-targeted therapy could be used as a novel upstream treatment of AF in the future.

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Disclosure Statement

All authors declare that they have no conflicts of interests.

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