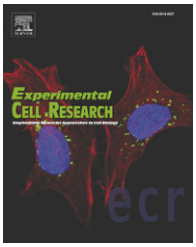
EXPERIMENTAL CELL RESEARCH 315 (2009) 1190–1199

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

Cx43 contributes to TGF-β signaling to regulate differentiation of cardiac fibroblasts into myofibroblasts

Yuko Asazuma-Nakamura[a,b,1], *Ping Dai*[a,1], *Yoshinori Harada*[a], *Yan Jiang*[a], *Kenji Hamaoka*[b], *Tetsuro Takamatsu*[a,*]

[a]*Department of Pathology and Cell Regulation, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan*

[b]*Department of Pediatric Cardiology and Nephrology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan*

ARTICLE INFORMATION

Article Chronology:
Received 3 September 2008
Revised version received 2 December 2008
Accepted 20 December 2008
Available online 6 January 2009

Keywords:
TGF-β
Connexin43
Antisense oligodeoxynucleotides
α-SMA
Cardiac fibroblast
Cardiac myofibroblast
Differentiation

ABSTRACT

Differentiation and activation of fibroblasts into myofibroblasts which express α-smooth muscle actin (α-SMA) are essential for wound healing and tissue repair. Change in fibroblast properties is initiated by transforming growth factor β (TGF-β). Here, we sought to investigate whether connexin43 (Cx43), a gap-junctional protein, contributes to differentiation of cardiac fibroblasts to myofibroblasts. In cultured neonatal rat cardiac fibroblasts, we found that expression of α-SMA increases in parallel with Cx43 by using immunocytochemistry, and that knockdown of the endogenous Cx43 activity with antisense oligodeoxynucleotides (AS) inhibits α-SMA expression significantly, while overexpression of Cx43 increases α-SMA expression remarkably. These findings demonstrate that Cx43 contributes to TGF-β signaling to regulate α-SMA expression. Thus, we propose a novel physiologic function of Cx43, which plays a critical role in the pathological activation of cardiac fibroblasts in the myocardial fibrosis associated with heart failure.

Introduction

About two thirds of the interstitial cells in the normal heart are fibroblasts, which actively cross talk with myocytes to determine the quantity and quality of extracellular matrix (ECM) [1,2]. Cardiac fibroblasts are a major constituent of the cardiac fibrosis associated with cardiac hypertrophy and heart failure. A main process in cardiac fibrosis is the differentiation of fibroblasts into myofibroblasts, which play a central role in connective-tissue remodeling and pathological wound healing [3]. During the transition of fibroblasts into myofibroblasts, the active remodeling process involves cytokine release and actin cytoskeleton and ECM synthesis, in sequential order [4]. Myofibroblasts are specialized contractile fibroblasts and characterized by *de novo* expression of α-smooth muscle actin (α-SMA) in response to potent fibrogenic cytokines [5].

It is known that the expression of α-SMA is initiated and promoted by transforming growth factor β (TGF-β) *in vivo* and *in vitro* [6–8]. TGF-β is a primary fibrogenic growth factor in heart failure [7] and functions as a key cytokine in the development of tissue fibrosis, including monocyte recruitment in the healing infarct and promoting granulation tissue formation in the inflammatory phase [9,10]. In addition, TGF-β markedly enhances collagen synthesis within the

* *Corresponding author.* Fax: +81 75 251 5353.
E-mail address: ttakam@koto.kpu-m.ac.jp (T. Takamatsu).
[1] These authors contributed equally to this work.

0014-4827/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2008.12.021

heart, and collagenous substrates induce discontinuous conduction between cardiomyocytes and fibroblasts [11].

Cardiac myofibroblasts are considered to originate from resident fibroblasts; bone marrow-derived fibroblasts and epithelial cells contribute to fibroblast accumulation through endothelial–mesenchymal transition/transformation (EMT), which is induced by TGF-β1 and prevented by bone morphogenic protein 7 (BMP-7) [12,13].

We have recently demonstrated that the activity of TGF-β is positively mediated by Cx43 through competitive binding to microtubules between endogenous Smads and Cx43 [14]. Moreover, Cx43 knockdown with either Cx43 antisense oligodeoxynucleotides (AS) or small interference RNA (siRNA) of Cx43 inhibits EMT in corneal endothelial injury [15]. Our previous findings led us to speculate that Cx43 can influence differentiation of myofibroblasts derived from cardiac fibroblasts. In this study, we used fibroblasts isolated from 2-day-old neonatal rat hearts and demonstrated that knockdown of the endogenous Cx43 activity with AS inhibits α-SMA expression, that overexpression of Cx43 increases α-SMA expression, and that Cx43 acts cooperatively with TGF-β to induce α-SMA expression. Our novel findings provide new insights into the mechanisms underlying the pathological activation of fibroblasts in cardiac fibrosis induced by TGF-β signaling.

Materials and methods

Primary cardiac fibroblast isolation and culture

Primary cardiac fibroblasts were isolated from 2-day-old neonatal Wistar rat hearts (Shimizu Laboratory Supplies Corp. Ltd., Kyoto, Japan). Briefly, ventricles were removed under sterile conditions from neonatal rats, placed in cold sterile calcium-free phosphate buffered saline (PBS), minced into approximately 2 mm cubes, and treated with 1 mg/ml collagenase (type II; Worthington Biochemical Corp., Lakewood, NJ). Dissociated cells were preplated for 45 min in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (FBS). This process was repeated twice, and cells were collected through trypsin (Sigma) treatment. The resulting cardiac fibroblasts were resuspended in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin and seeded in standard culture dishes for immunostaining and luciferase reporter assays. All fibroblasts used in our experiments were at passage 2. All animal experiments were conducted with the approval of and in accordance with guidelines from the Committee for Animal Research, Kyoto Prefectural University of Medicine.

Immunocytochemistry and image analysis

Fibroblasts were seeded into 12-well plates overlaid with cover glasses and grown to 50% confluence in DMEM containing 0.2% FBS. For TGF-β treatment, cells were treated with or without 5 ng/ml TGF-β1 (Sigma) dissolved in 1 mg/ml of bovine serum albumin containing 4 mM HCl for different lengths of time, as indicated in the legends for Figs. 1 and 5A. For TβRI/ALK5 inhibitor treatment, cells were treated with or without 10 μM SB-431542 (Tocris, Ballwin, MO) dissolved in dimethyl sulfoxide (DMSO) (Sigma) for different lengths of time, as indicated in the legends for Figs. 2 and 4B. For Cx43 knockdown and Cx43 overexpression, transient transfection of fibroblasts was carried out using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To knockdown Cx43 activity, cells were transfected with 20 ng of pEGFP-N1 (Clontech, Mountain View, CA), which served as a transfectant indicator, and either 1 μg of AS to Cx43 (5′-GTAATTGCGGCAGGAGGAATTGTTTCTGTC-3′) [16] or 1 μg of sense oligodeoxynucleotides (S) to Cx43 (5′-GACAGAAACAATTCC-TCCTTGCCGCAATTTAC-3′) [16]. To overexpress Cx43, cells were transfected with 1 μg of Cx43-EGFP [17]. Cells were rinsed in ice-cold PBS and fixed in 2% paraformaldehyde in PBS for 30 min. After fixation, cells were permeabilized by incubation with 0.1% Triton X-100 in PBS for 10 min. Cells were then blocked with 3% skim milk in PBS for 1 h. Primary antibodies used were monoclonal anti-α-SMA antibody (clone 1A4, A2547; Sigma) (1:5000) and rabbit anti-Cx43 polyclonal antibodies (71-0700; Zymed Laboratories, South San Francisco, CA) (1:500). Secondary antibodies were used with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen-Molecular Probes, Eugene, OR) (Figs. 1 and 2) or Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen-Molecular Probes) (Fig. 4) and Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen-Molecular Probes) (Figs. 4 and 5), and DNA was used with TO-PRO-3 iodide (Invitrogen-Molecular Probes). Cells were washed carefully with PBS (5 times, 10 min each time) after incubation with primary and secondary antibodies. After immunostaining, samples were mounted and analyzed by confocal microscopy (FV1000; Olympus, Tokyo, Japan).

Fluorescence intensities were quantified by using FV10-ASW software (Olympus).

Luciferase reporter assays

Fibroblasts were seeded at a density of 5×10^5 into 3.5-cm dishes. The next day, cells were transfected with 100 ng of the internal control plasmid pRL-tk, and either 200 ng of human α-SMA luciferase reporter plasmid p895-Luc (p895-Luc) [18] or 200 ng of the empty vector pGL3-Luc (Promega, Madison, WI). For TGF-β treatment, after transfection, cells were incubated for 12 or 32 h in DMEM containing 10% FBS, followed by inductions for 8 h with or without 5 ng/ml TGF-β1. For TβRI/ALK5 inhibitor treatment, cells were incubated for 20 or 40 h in DMEM containing 10% FBS and either DMSO or 10 μM SB-431542. For Cx43 knockdown, cells were transfected with 100 ng of pRL-tk, 200 ng of p895-Luc, and either 1 μg of AS to Cx43 or 1 μg of S to Cx43. As a control, cells were transfected with 100 ng of pRL-tk, 200 ng of pGL3-Luc, and 1 μg of the nonsense sequence control oligodeoxynucleotides (C) (5′-AATTCTCCGAACGTGTCACGT-3′) [19]. For Cx43 overexpression, cells were transfected with 100 ng of pRL-tk, 200 ng of p895-Luc, and 1 μg of pcDNA.3.1.Cx43 [14]. For luciferase assays, all samples were harvested together at 40 h after transfection. Luciferase activity in cell lysates was measured using the dual luciferase assay system (Promega) in a Berthold Lumat LB 9705 luminometer (Pforzheim, Germany).

Statistical analysis

Data are expressed as the mean ± SD. Differences between groups were evaluated by Student's *t*-test. Differences of $p<0.05$ were considered statistically significant. For all experiments, at least 3 replicates were included, and all experiments were repeated at least 3 times.