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Pathophysiological roles of canstatin on myofibroblasts after myocardial infarction in rats

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ABSTRACT

Myofibroblasts play an important role during remodeling process after myocardial infarction through proliferation, migration, production and degradation of extracellular matrix (ECM) and contraction. Canstatin, a 24 kDa polypeptide, is cleaved from α2 chain of type IV collagen, which is a major component of basement membrane around cardiomyocytes. We examined the effects of canstatin on myofibroblasts isolated from the areas of myocardial infarction. Myocardial infarction model was made by ligating left anterior descending artery of Wistar rats. Two weeks after the operation, the cells were isolated by an explant method and identified as myofibroblasts with immunofluorescence staining. Cell counting assay was performed to examine cell proliferation. Boyden chamber assay was performed to examine cell migration. Expression and phosphorylation of proteins were detected by Western blotting. Collagen gel contraction assay was performed to measure cell contractility. Canstatin stimulated proliferation, secretion of matrix metalloproteinases, expression of cyclooxygenase (COX)-2, and inhibited collagen gel contraction in myofibroblasts. Canstatin increased Akt phosphorylation. LY294002, a phosphoinositide-3-kinase/Akt inhibitor, inhibited the canstatin-induced proliferation. NS-398, a COX-2 inhibitor, suppressed the inhibitory effect of canstatin on collagen gel contraction. Canstatin expression in areas of myocardial infarction 2 weeks after surgery decreased. We for the first time demonstrate that canstatin is an endogenous bioactive molecule regulating the various functions of myofibroblasts after myocardial infarction. The decrease of canstatin expression in the maturated areas of myocardial infarction might lead to stabilization of scar tissues perhaps in part through the reduction of proliferation and ECM degradation as well as the stimulation of contractility in myofibroblasts.

1. Introduction

Myocardial infarction evoked by coronary artery occlusion is one of the leading causes of death among cardiovascular diseases. Within 30 min after acute myocardial infarction, irreversible cardiomyocyte death is occurred by ischemia and the acute inflammatory responses, such as release of inflammatory mediators and production of matrix metalloproteinases (MMPs) which degrade extracellular matrix (ECM) (Ma et al., 2014, 2013). Because cardiomyocytes have poor regenerative capacity, the area of infarction is replaced with a fibrous tissue containing various ECMs including type I collagen (Ma et al., 2014, 2012b). After myocardial infarction, fibroblasts are stimulated with transforming growth factor (TGF)-β and differentiate into myofibroblasts which are the major source of ECM (Ignotz and Massague, 1986). Myofibroblasts are involved in wound healing of the areas of infarction by migration, proliferation and secretion of collagens (mainly type I and III) (Hinz, 2007; Ma et al., 2014; van Putten et al., 2016). Because myofibroblasts highly express α-smooth muscle actin (α-SMA), they contract and stabilize the scar tissues (Hinz et al., 2001; Tomasek et al., 2002). Transient activation of myofibroblasts after myocardial injury is beneficial and essential to maintain the structure of the heart, while an insufficient scar formation causes left ventricular dysfunction and rupture. On the other hand, chronic and persistent activation of myofibroblasts induces pathological ECM accumulation and remodeling, which causes cardiac dysfunction (Ma et al., 2014, 2012b).

Type IV collagen, a major component of basement membrane, is ubiquitously expressed around cardiomyocytes (Prockop and Kivirikko, 1995; Timpl, 1996). Canstatin, a 24 kDa polypeptide, is cleaved from non-collagenous 1 domain of α2 chain. Canstatin is considered to bind its receptor, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins and exhibit anti-angiogenic effect in vascular endothelial cells (Hwang-Bo et al., 2012; Kamphaus et al., 2000; Magnon et al., 2005) and anti-tumor effect through the induction of tumor cell apoptosis (Xing et al., 2014). Therefore, canstatin is expected as a novel endogenous anti-tumor factor.

The expression of α1 and α2 chains of type IV collagen increases in

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the area of myocardial infarction in rats (Murakami et al., 1998). It has also been reported that MMP-2 and MMP-9, a major catabolizing enzyme of type IV collagen, increased during myocardial infarction (Deten et al., 2003; Romanic et al., 2001). Therefore, it is hypothesized that canstatin, a cleaved product of type IV collagen, plays a role during remodeling process of the myocardial infarction. Recently, we reported that canstatin exhibited a protective effect on isoproterenol-induced apoptosis and hypoxia-induced apoptosis in H9c2 cardiomyoblasts (Okada et al., 2016; Kanazawa et al., 2017). Thus, it is suggested that canstatin has different physiological activity on heart tissues compared with other tissues. We also reported that canstatin stimulated migration of adult rat cardiac fibroblasts (Okada et al., 2017). However, the effect of canstatin on biological activities of myofibroblasts has not been clarified. In this study, we thus investigated the effects of canstatin on proliferation, migration, ECM production, contraction and expression of MMPs and cyclooxygenase (COX)−2 of myofibroblasts, which are reported to be involved in cardiac remodeling (Iyer et al., 2012; Saito et al., 2000; Wong et al., 1998).

2. Materials and methods

2.1. Reagents and antibodies

Reagent sources were as follows: recombinant mouse collagen alpha-2 (IV) chain, partial (canstatin) (Cusabio Biotech, Wuhan, Hubei, China), LY294002 (Wako, Osaka, Japan), NS-398 (Cayman Chemical, Ann Arbor, MI, USA), prostaglandin E_2 (PGE_2) (Sigma-Aldrich, St. Louis, MO, USA) and recombinant human interleukin (IL)-1β (Peprotech, Rocky Hill, NJ, USA). Canstatin used in this study is the C-terminal domain of collagen alpha-2 (IV) chain of mouse. The amino acid sequence was 99% identity with that of rats (accession: EDM08821, 1476–1695) and the sequence was as follows: VKHSQTDQEPMCPVGMNKLWSGYSLLYFEGQEKAHNQDLGLAGSC-LARFSTMPFLYCNPGDVCYYASRNDKSYWLSTTAPLPMMPVAEEEI-KPYISRCSVCEAPAVAIAVHSQDTSIPHCPAGWRSLWIGYSFLMHTA-AGDEGGGQSLVSPGSCLEDFRATPFIECNGGRGTCHYFANKYSFWL-TTIPEQNFQSTPSADTLKAGLIRTHISRCQVCMKNL.

Antibodies sources were as follows: anti-phospho-Akt (Ser473), anti-total Akt (tAkt), anti-phospho-extracellular signal-regulated kinase (ERK) (Cell Signaling Technology, Beverly, MA, USA), anti-total ERK1 (tERK1), anti-COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-collagen type I (Rockland Immunochemicals, Gilbertsville, PA, USA), anti-MMP-2 (Kyowa pharma chemical, Toyama, Japan), anti-MMP-9 (EMD Millipore, Billerica, MA, USA), anti-total actin, anti-vimentin (Sigma-Aldrich), anti-CD31, anti-α-SMA (DAKO, Glostrup, Denmark), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Wako), anti-canstatin (Boster Biological Technology, Pleasanton, CA, USA), anti-rabbit IgG horseradish peroxidase linked whole antibody, anti-mouse IgG horseradish peroxidase linked whole antibody (Amersham Biosciences, Buckinghamshire, UK and Cell signaling Technology), anti-goat IgG horseradish peroxidase linked whole antibody (Sigma-Aldrich), Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA).

2.2. Myocardial infarction model

All animal studies were approved by the President of Kitasato University through the judgment by Institutional Animal Care and Use Committee of Kitasato University (Approval no. 15–158 and 16–032). Adult male Wistar rats (7–10-week old; CLEA Japan, Tokyo, Japan) were cared in accordance with the National Institutes of Health Guide for the Care of Laboratory Animals and the guideline for animal care and treatment of the Kitasato University. Myocardial infarction was induced by coronary artery ligation as described previously (Wu et al., 2011). After the rats were anesthetized with isoflurane, endotracheal intubation was performed and they were mechanically ventilated with isoflurane. The operating field (chest area) was treated with depilatory cream for hair removal and disinfected with ethanol and chlorhexidine gluconate. Left thoracotomy was performed between third or fourth intercostal space, and heart was exposed with a small incision of pericardium. Then, the proximal left anterior descending artery was permanently ligated using a 6-0 nylon suture. After the chest was closed, rats were recovered and buprenorphine (0.005 mg/100 g) was subcutaneously administered. Sham operated rats were made by the same method except for ligation.

2.3. Isolation of myofibroblasts from the areas of myocardial infarction

The myocardial infarction model rats were cared for 2 weeks and the hearts were isolated under anesthesia with intraperitoneal injection of pentobarbital (100 mg/kg). The isolated heart was washed with oxygenated Krebs-Henseleit solution (119 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 10.0 mM Glucose) and the infarcted region (in the case of sham-operated group, the left ventricular tissue correlated to the infarcted region) was isolated. The infarcted region was immediately frozen for protein extraction with liquid nitrogen and preserved at −80 °C. The infarcted region was cut into 4–5 mm pieces for isolation of myofibroblasts and washed five times with sterilized Tris-buffered saline (TBS, pH 7.4) and five times with Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich) containing 10% fetal bovine serum (FBS, Gibco/Life technologies, Carlsbad, CA, USA and HyClone/GE Healthcare, Little Chalfont, UK). Then, the piece of tissue was put on 100 mm culture dish with DMEM containing 10% FBS and incubated at 37 °C in 5% CO_2. Medium was changed every 2–3 days. After 10 days of culture, the piece of tissue was removed and the migrated and proliferated cells around the tissue were trypsinized and passaged. *Passage* 2–8 cells were used as areas of infarction-derived myofibroblasts. A 24 h starvation in DMEM without FBS was performed before the stimulation.

2.4. Immunofluorescence staining

Immunofluorescence staining was performed to identify the isolated cells as myofibroblasts as described previously (Iwanaga et al., 2009). The cells cultured on 6 well-culture plate were fixed with 4% paraformaldehyde at 4 °C for 10 min. After permeabilized with 0.2% Triton X-100, the cells were incubated with 5% normal goat serum. They were incubated with mouse anti-α-SMA antibody, mouse anti-vimentin antibody, mouse anti-CD31 antibody or rabbit anti-collagen type I antibody at 4 °C overnight. After they were incubated with anti-mouse Alexa 488 dye-conjugated IgG or anti-rabbit Alexa 568 dye-conjugated IgG at room temperature for 1 h, the nuclei were stained with 4′,6-Diamidino-2-phenylindole (DAPI) solution (Dojindo, Kumamoto, Japan) for 5 min at room temperature. The images were captured using a CCD camera (MicroPublisher 5.0 RTV)-equipped fluorescence microscope (BX-51; OLYMPUS, Tokyo, Japan).

2.5. Phase contrast microscopy

Myofibroblasts were grown to confluent in 6-well culture plate and stimulated with canstatin (10–250 ng/ml) or IL-1β (10 ng/ml) for 48 h. Control group was treated with vehicle of canstatin (20 mM Tris, 500 mM L-arginine, 50% glycerol, pH 8.0). Cell morphology was observed with a phase contrast microscope (CKX-31, OLYMPUS).

2.6. Cell counting assay

Cell counting assay was performed by using Cell counting kit-8 (Dojindo) as described previously (Okada et al., 2015). Myofibroblasts were grown to 50–60% confluence in 6-well culture plate and