



Immunohistochemical characterization of myofibroblasts appearing in isoproterenol-induced rat myocardial fibrosis

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ABSTRACT. Fibrotic lesion is formed by myofibroblasts capable of producing collagens. The myofibroblasts are characterized by immunoexpressions of vimentin, desmin and α -smooth muscle actin (α -SMA) in varying degrees. The cellular characteristics remain investigated in myocardial fibrosis. We analyzed immunophenotypes of myofibroblasts appearing in isoproterenol-induced myocardial fibrosis in rats until 28 days after injection (10 mg/kg body weight); the lesions developed as interstitial edema and inflammatory cell reaction on 8 hr and days 1 and 3, and fibrosis occurred on days 1, 3, 7, 14, and 21 by gradual deposition of collagens, showing the greatest grade on day 14; the lesions gradually reduced with sporadic scar until day 28. Myofibroblasts expressing vimentin and α -SMA increased with a peak on day 3, and then, gradually decreased onwards. Interestingly, Thy-1 expressing cells appeared in the affected areas, apparently being corresponding to the grade similar to vimentin- and α -SMA-positive cells. Thy-1 is expressed in immature mesenchymal cells such as pericytes with pluripotent nature. The immunoreactivity for A3-antigen, a marker for immature mesenchymal cells, was seen in some surrounding cells. There were no cells reacting with antibodies to nestin or glial fibrillary acidic protein, although hepatic myofibroblasts have been reported to react with these antibodies. Collectively, myofibroblasts appearing in rat myocardial fibrosis may have been derived from immature mesenchymal cells positive for Thy-1 or A3-antigen, with thereafter showing expressions of vimentin and α -SMA in differentiation.

KEY WORDS: immunohistochemistry, myocardial fibrosis, myofibroblasts, rat

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Myofibroblasts are the main cell capable of producing collagens, and the cells contribute to reparative fibrosis after tissue injury as a healing process. On the contrary, intractable fibrosis such as liver cirrhosis and atrophic kidneys lead to organ insufficiency; the detail mechanisms of how the myofibroblasts participate in should be investigated [5, 7, 10]. The myofibroblasts are generally considered to originate from pre-existing fibroblasts [16]. Along with the fibroblasts, hepatic stellate cells (HSCs) expressing glial fibrillary acidic protein (GFAP) can be transformed into myofibroblasts in hepatic fibrosis [13, 18, 23], and myofibroblasts formed via the epithelial-mesenchymal transition (EMT) of regenerating renal tubular epithelial cells take part in renal interstitial fibrosis [17]. Furthermore, immature mesenchymal cells which may have pluripotential differentiations may be related to the origin [4, 9, 15]. The original cells of myofibroblasts look heterogeneous; the cells are still regarded as mysterious cells. Pathologically, the myofibroblasts have been identified by using immunohistochemistry with antibodies to vimentin, desmin and α -smooth muscle actin (α -SMA) [3, 18].

The properties of myofibroblasts in hepatic and renal fibrosis have been well described [4, 9, 13, 15], whereas those in myocardial fibrosis remain to be analyzed. The myocardial fibrosis usually occurs after tissue damage due to infarction, cardiomyopathy and valvular diseases [12]. Extensive fibrosis in myocardium results in death, whereas myocardial fibrosis in a case which is not so severe may lead to cicatrix lesion or complete recovery [2]. The immunophenotypical characterization and possible origin of myofibroblasts appearing in the myocardial fibrosis should be investigated, in comparison with those in fibrosis in liver and kidneys.

Isoproterenol, a potent beta adrenergic agonist with peripheral vasodilator, bronchodilator, and cardiac stimulating properties, has been used for treatments for bradycardia, atrioventricular block and bronchial asthma [14]. On the other hand, excessive dose

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Table 1. Primary antibodies used for immunohistochemistry

Antibody	Clone	Type	Dilution	Pre-treatment	Source	Specificity
Vimentin	V9	Mouse monoclonal	1:500	Microwave in citrate buffer, 20 min	Dako Corp, Glostrup, Denmark	Cells of mesenchymal origin
Desmin	D33	Mouse monoclonal	1:200	No	Dako Corp.	Smooth muscle cells, Ito cells (rat)
α -SMA	1A4	Mouse monoclonal	1:1,000	Microwave in citrate buffer, 20 min	Dako Corp.	Smooth muscle cells, myofibroblasts
Thy-1	CD90	Mouse monoclonal	1:500	Microwave in citrate buffer, 20 min	Cedarlane Laboratories Ltd., Ontario, Canada	Immature mesenchymal cells
GFAP	-	Rabbit polyclonal	1:500	10 μ g/ml proteinase K, 10 min at 37°C	Dako Corp.	Astroglial cells
Nestin	Rat-401	Mouse monoclonal	1:200	Microwave in citrate buffer, 20 min	Millipore, Temecula, CA, U.S.A.	Neuroepithelial stem cells and activated HSCs
MFH	A3	Mouse monoclonal	1:500	Microwave in citrate buffer, 20 min	TransGenic Inc., Kobe, Japan	Mesenchymal stem cells

A3 antibody was generated by using immature mesenchymal cell-derived malignant fibrous histiocytoma (MFH) as the antigen.

of isoproterenol is experimentally used to induce myocardial fibrosis in rats and mice, resulting from myocardial necrosis [20]. The purpose of this study is to determine the properties of myofibroblasts appearing in fibrotic lesions in rats administered with isoproterenol.

MATERIALS AND METHODS

Animals and experimental procedures

Six-week-old male Sprague-Dawley (SD) rats were purchased from Japan SLC (SLC, Shizuoka, Japan). These animals were maintained in a room at $21 \pm 3^\circ\text{C}$ with a 12 hr light-dark cycle, and they were fed a standard diet for rats (F-2; Funabashi Farm, Funabashi, Japan) and supplied with tap water *ad libitum*. After one week acclimatization, twenty-four animals were given a single subcutaneous injection of isoproterenol (10 mg/kg body weight in saline solution, 5 ml/kg) [20]. Animals were euthanized by exsanguination under deep isoflurane anesthesia on 8 hr, and 1, 3, 5, 7, 14, 21, and 28 days after the injection (n=3 in each). The remaining 3 rats, that received saline injection (5 ml/kg), severed as controls and were sacrificed on day 1. Animal housing and sampling conformed to the institutional guidelines approved by the ethics committee of Nippon-Sinyaku Co., Ltd. for the Care and Use of Experimental Animals (No. B3042202).

Histopathology and immunohistochemistry

Myocardium tissues were fixed in 10% neutral buffered formalin or fixed in periodate-lysine-paraformaldehyde (PLP) solutions [6]. Deparaffinized sections that were cut at 4 μ m in thickness were stained with hematoxylin and eosin (HE) for morphology and with Masson's trichrome for collagen deposition.

Deparaffinized PLP-fixed sections were immunostained with primary antibodies listed in Table 1. After primary antibody treatment, the sections were incubated with horseradish peroxidase-conjugated secondary antibody (Histofine simplestain MAX-PO, Nichirei, Tokyo, Japan) for 30 min. Positive reactions were visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB substrate kit, Nichirei), and the sections were lightly counter-stained with hematoxylin. As negative controls, tissue sections were treated with mouse or rabbit non-immunized serum instead of the primary antibody.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

A part (3 mm³) of myocardium samples obtained from the subendocardial portions of the left ventricles, which the lesions occurred frequently, were immersed in RNA later (Qiagen GmbH, Hilden, Germany) and stored at -80°C until use. Total RNA was extracted using SV total RNA isolation system (Promega, Madison, WI, U.S.A.). The concentration of RNA was determined by using a nanodrop 1000TM spectrophotometer (Thermo Scientific, Wilmington, DE, U.S.A.) and 2.5 μ g of total RNA was reverse-transcribed to cDNA using Superscript VILO reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). Real-time PCR for transforming growth factor- β 1 (TGF- β 1; Assay ID, Rn 00572010_m1) was conducted by using TaqMan gene expression assays (Life Technologies, Carlsbad, CA, U.S.A.) in a PikoReal Real-time PCR System (Thermo Scientific). The mRNA expression was normalized against that of 18s rRNA (Assay ID: Hs99999901_s1) as an internal control. The data were calculated using the comparative C_t method ($^{\Delta\Delta}C_t$ method) [22].

Histological and data evaluation

In HE- and Masson's trichrome-stained sections, the degree of myocardial fibrosis was evaluated by grading as shown in Table 2 [8]: -, normal without fibrosis; \pm , very slight fibrosis; +, slight fibrosis; ++, moderate fibrosis; +++, severe fibrosis with diffuse

Table 2. Semiquantitative evaluation of myocardial fibrosis and immunopositive cells reacting to vimentin, α -smooth muscle actin (α -SMA) and Thy-1 in the affected arear in isoproterenol-administered rats

	Control	8 hr	Day 1	Day 3	Day 5	Day 7	Day 14	Day 21	Day 28
Fibrosis	–	–	±	+	++	++	+++	+~++	+~++
Antibody									
Vimentin	–	±	±~+	++~+++	+	+	+~++	–~±	–
α -SMA	–	–	–~±	+~++	+	±~+	±~+	–~±	–~±
Thy-1	±	±	±	++	++	++	+~++	+	+

Fibrosis was evaluated as follows; –, normal without fibrosis; ±, very slight fibrosis; +, slight fibrosis; ++, moderate fibrosis; and +++, severe fibrosis with diffuse lesion. Grading of immunopositive cells was evaluated as follows (approximate numbers of positive cells counted at $\times 400$): –, no immunopositive cells; ±, a few immunopositive cells (less than 10 cells); +, a small number of immunopositive cells (10–40 cells); ++, a moderate number of immunopositive cells (41–100 cells); and +++, a large number of immunopositive cells (more than 100 cells).

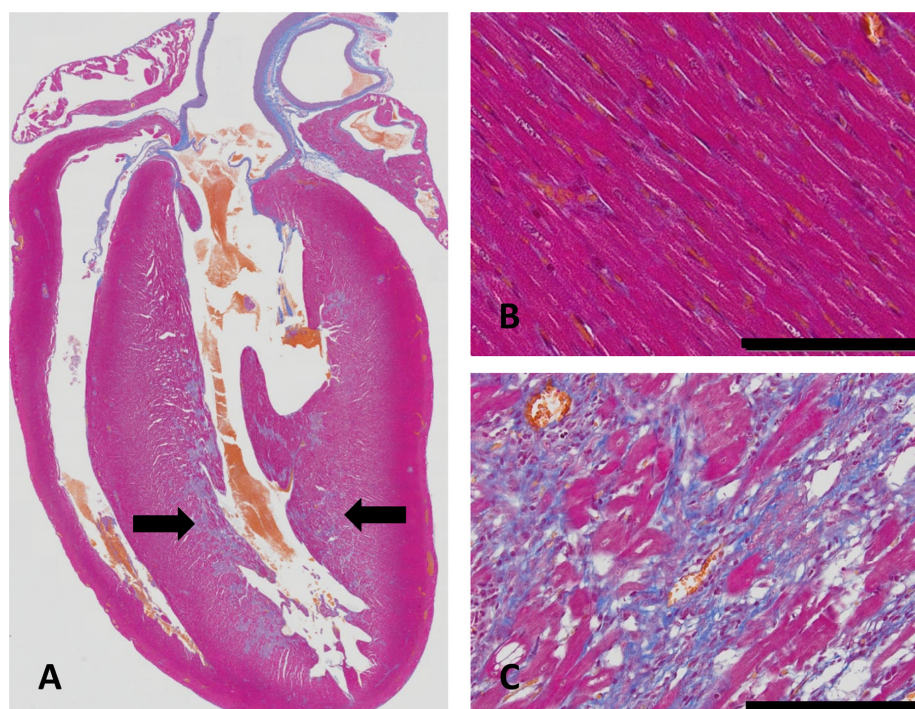


Fig. 1. A: Histopathology of isoproterenol-induced rat myocardial fibrosis on day 5 (fibrosis grade ++ after injection); the lesions are seen in subendocardial portions of the left ventricle (arrows). B: Normal myocardial structure in a control rat is shown. C: Fibrotic lesion characterized by collagen fiber deposition is seen on day 5. A, B, C, Masson's trichrome stain used for collagens (blue); A, loupe magnified image; B and C, bar=200 μ m.

lesion. The immunopositive cells seen in myocardial fibrosis were evaluated semi-quantitatively as follows (approximate numbers of positive cells counted at $\times 400$) [8]: –, no immunopositive cells; ±, a few immunopositive cells (less than 10 cells); +, a small number of immunopositive cells (10–40 cells); ++, a moderate number of immunopositive cells (41–100 cells); and +++, a large number of immunopositive cells (more than 100 cells) (Table 2). Quantitative data for TGF- β 1 mRNA expression were shown as mean \pm standard deviation (SD), and statistical analysis was performed using Dunnett's multiple comparison tests; significance was considered at $P < 0.05$.

RESULTS

Histopathology

Isoproterenol-induced myocardial injury occurred exclusively in the subendocardial portions of the left ventricles (Fig. 1A). The lesion was characterized by necrotic myocytes, inflammation and subsequent reparative fibrosis; myocyte necrosis and edema were seen on 8 hr and day 1; thereafter, inflammatory cells appeared on days 1 to 21 with a peaked cellularity on day 3, consisting mainly of macrophages (data not shown). Collagen deposition was not seen in control myocardium (Fig. 1B); slight collagen deposition was present on 8 hr (\pm), and then, distinct fibrosis, accompanied by gradual collagen fiber deposition (demonstrable blue

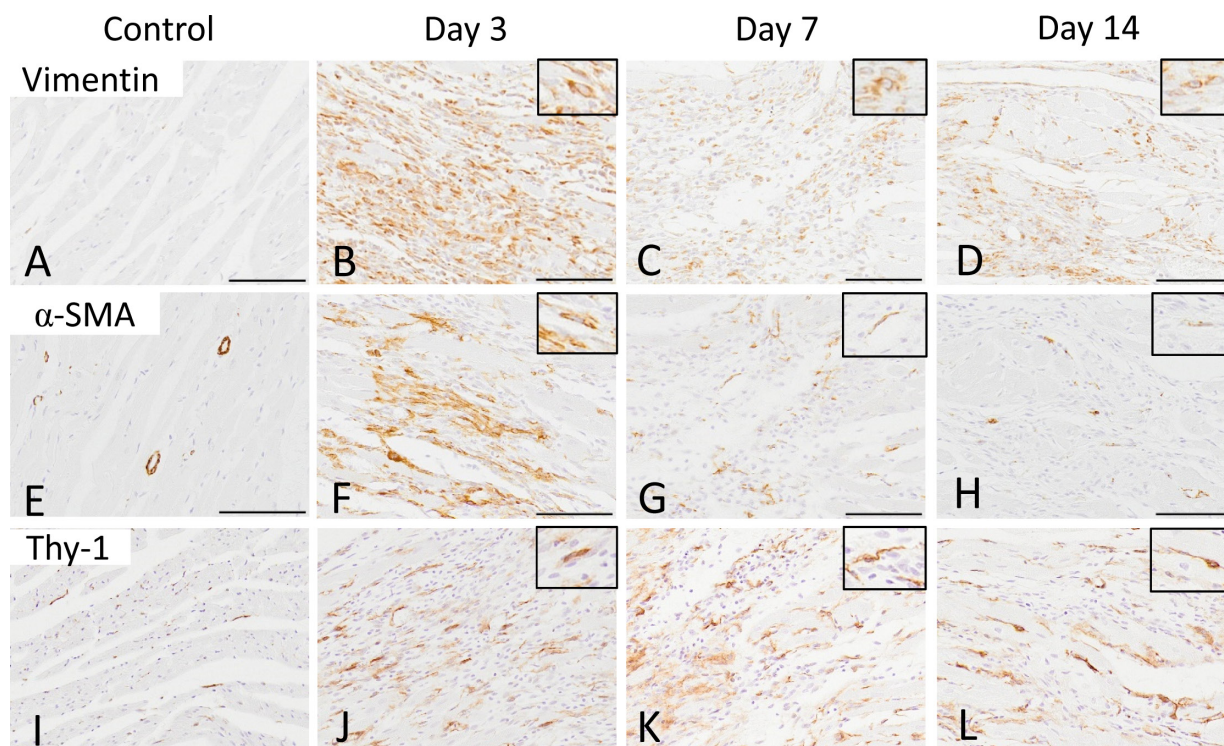


Fig. 2. Distribution of cells reacting to vimentin (A–D), α -smooth muscle action (α -SMA) (E–H) and Thy-1 (I–L) in control (A, E, I) and isoproterenol-induced fibrotic areas on days 3 (B, F, J) (fibrosis grade +), 7 (C, G, K) (fibrosis grade ++) and 14 (D, H, L) (fibrosis grade +++). Although vascular smooth muscles reacting to α -SMA and a few Thy-1-positive interstitial cells are seen in controls, increased numbers of myofibroblasts reacting to vimentin, α -SMA and Thy-1 are seen in varying degrees (as shown in Table 2) in the affected areas on days 3, 7, and 14, and these cells exhibit mainly spindle-shaped configuration. Immunohistochemistry, counterstained with hematoxylin; bar=100 μ m; inset, a higher magnification of each positive cells.

with Masson's trichrome stain; Fig. 1A, 1C), developed on days 1 to 21, showing a peak on day 14 (+++) (Table 2). On day 28, the myocardial injury almost recovered, but there were some focal scars in the affected areas (+ ~ ++).

Immunoreactivity for vimentin, desmin, and α -SMA

In the affected areas, cells reacting to vimentin and α -SMA were seen, and these cells were spindle-shaped or oval in shape (Fig. 2). The vimentin-positive cells (Fig. 2A–D) began to appear at 8 hr in the edematous interstitial areas (\pm), and gradually increased on days 1 and 3 with a peak on day 3 (++ ~ +++); on day 5 onwards, the grade gradually decreased and returned to that in controls on day 28 (Table 2). α -SMA-positive cells (Fig. 2E–H) began to increase on day 1 (\pm), and showed a peak on day 3 (++); subsequently, the grade gradually decreased until day 28 (\pm), but not completely recovered to control levels. Desmin immunoreactivity was seen in surrounding normal myocytes as specific reaction. Therefore, it was difficult to distinguish desmin-positive injured myocytes and myofibroblasts. Desmin immunoreactivity could not be evaluated in this model.

Immunoreactivity for Thy-1, GFAP, nestin and A3-antigen

Thy-1-positive cells slightly appeared in the affected, edematous areas on 8 hr and day 1 (\pm), and thereafter, quickly increased on day 3 (++) with retained grade until day 14 (Fig. 2I–L); the Thy-1-positive cells showed spindle-shaped configuration. In controls, there were a few Thy-1-positive cells in the myocardium (\pm); Thy-1-positive cells were present exclusively around blood vessels (Fig. 3A); such pericytes were seen in the surrounding tissues of the affected myocardium with greater reactivity.

There were no cells reacting to GFAP or nestin. Interestingly, cells positive for A3-antigen increased slightly in the surrounding tissue of the affected areas on days 3–14. The grade was not so severe as evaluation for other mesenchymal markers; apparently, these cells were regarded as capillary-constituting cells and some interstitial cells (Fig. 3B).

TGF- β 1 mRNA expression

As compared with that of controls, mRNA expression of TGF- β 1 was significantly increased as early as 8 hr, and the increased level also showed a statistical significance on days 1 and 28. These findings at least showed an occasional tendency of TGF- β 1 mRNA expression to increase after the injection (Fig. 4).

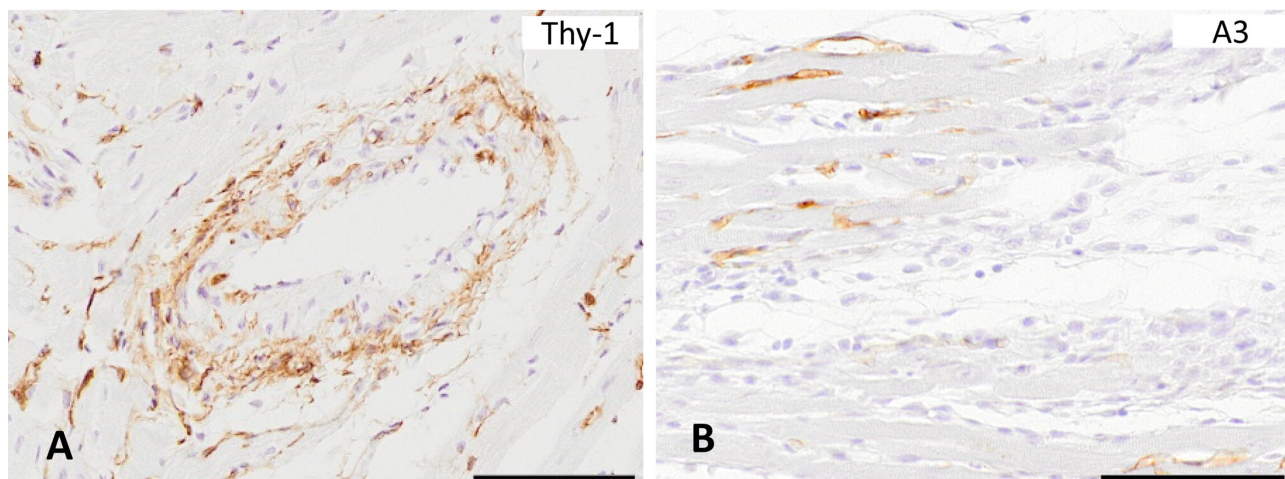


Fig. 3. Immunohistochemistry for Thy-1 on day 14 (A) (fibrosis grade +++) and for A3-antigen on day 7 (B) (fibrosis grade ++) in isoproterenol-induced myocardial lesions. In addition to Thy-1-positive myofibroblasts shown in Fig. 2 (J, K, L), increased number of Thy-1-positive cells are seen in blood vessels in the surrounding tissues of myocardial fibrosis; these cells are regarded as pericytes with pluripotency. The immunoreactivity for A3-antigen is seen in capillary and some spindle-shaped cells in surrounding tissues of the myocardial cells; the immunoreactivity for A3-antigen has been reported to be seen in immature cells in newly-formed blood vessels in lesions and bone marrow stem cells. Immunohistochemistry, counterstained with hematoxylin; bar=100 μ m.

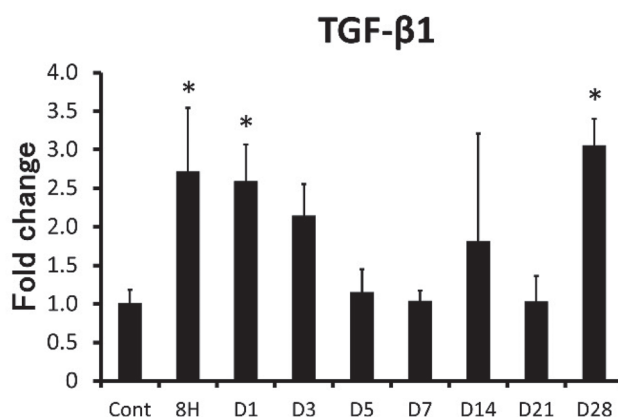


Fig. 4. mRNA expressions of transforming growth factor- β 1 in control (Cont) and myocardial tissues on 8 hr (H) and 1, 3, 5, 7, 14, 21, and 28 days (D) after isoproterenol injection. Asterisks, $P < 0.05$ by Dunnett's multiple test.

DISCUSSION

Myocardial fibrosis is characterized by myocyte necrosis and inflammation, followed by fibrosis [12, 14]. Isoproterenol-induced myocardial lesions showed a series of process of myocardial fibrosis as described previously [14, 20]; the fibrotic lesions with collagen deposition increased with a peak on day 7, and then, decreased until day 29, indicating reparative fibrosis in the present rat model.

In fibrotic lesions in liver and kidneys, it is well known that myofibroblasts appear by producing collagens, resulting in fibrosis [5, 7, 9, 18]. The cells were characterized by immunoexpressions of vimentin, desmin and α -SMA [11, 18]. In isoproterenol-induced myocardial fibrosis, although desmin-expression was not evaluated, cells reacting to vimentin and α -SMA were confirmed; the kinetics of these cells was corresponding generally to the degree of fibrotic lesion development as shown in Table 2. These findings indicated that myofibroblasts participate in development of myocardial fibrosis, as well.

Myofibroblasts is heterogeneous in the origin [4, 9]. Pre-existing interstitial fibroblasts have been considered to be the main cells capable of differentiating toward myofibroblasts by obtaining cytoskeletons such as vimentin, desmin and α -SMA in various degrees; out of them, α -SMA expression is the most important for identification, because myofibroblasts have nature between fibroblasts and smooth muscle cells, thereby being called "contractile cells" [11]. In cutaneous wound healing, pericytes and connective tissue sheath cells of hair follicles may differentiate toward myofibroblasts, and these cells reacted to Thy-1; in addition,

these cells are regarded as immature mesenchymal cells with totipotency [11, 24]. In the present myocardial fibrosis, Thy-1-positive cells appeared in the affected areas, with the grade and distribution similar to those of vimentin- and α -SMA-positive cells. Generally, pericytes react to Thy-1 as observed also in the present study (Fig. 3A). Thy-1-positive cells seen in the affected areas might be recruited from such pericytes, and then, expressed myofibroblastic nature, as discussed in cutaneous fibrosis [11].

It has been reported that there are myofibroblasts reacting to nestin (that is expressed in primitive ectodermal cells) and GFAP (a type III intermediate filament protein that was originally found as a marker for astrocytes) in experimentally-induced hepatic fibrosis [1, 18]. Because HSCs can express GFAP and nestin at immature stages, HSCs are considered to be the main cellular origin of myofibroblasts in hepatic fibrosis [18]. Recently, we reported that GFAP-positive pancreatic stellate cells could participate in pancreatic fibrosis in dogs and cats [8]. However, there were no cells reacting to GFAP or nestin in the present myocardial fibrosis.

Furthermore, there may be myofibroblasts which are formed via the EMT in which regenerating renal epithelial cells after injury can transform into mesenchymal cells, resulting in progressive renal interstitial fibrosis [9, 18]. Because epithelial elements are not present in the myocardium, the EMT is not responsible for myofibroblast formation in the present myocardial fibrosis.

In addition to the cellular origins as mentioned above, last decade, bone marrow stem cells have been considered to participate in fibrosis through possible transdifferentiation of endothelial cells, pericytes and immature interstitial mesenchymal cells towards myofibroblasts [9, 11, 17]; such stem cells are called “Muse cells (multi-lineage differentiating stress enduring cells)” [19]. Bone marrow stem cells, endothelial cells and pericytes react to A3-antigen and Thy-1 [11, 18]. The A3 antibody was generated by using immature mesenchymal cell-derived malignant fibrous histiocytoma (MFH) as the antigen [11, 18]. It is interesting to investigate relationship between myofibroblasts and “Muse cells” in future studies, because there were cells reacting to A3-antigen and Thy-1 in the present rat myocardial fibrosis.

It is well known that myofibroblast formation is regulated by TGF- β 1 produced by inflammatory cells, particularly M2 macrophages [21]. M2 macrophages expressing CD163 increased in the later observation period (on days 7–28) in the present myocardial fibrosis; characterization of macrophage phenotypes in this model is in progress. The occasional increase of TGF- β 1 mRNA level seen on 8 hr as well as days 1 and 28 might be responsible for myofibroblast development.

In short, the present study showed that myofibroblasts reacting to vimentin and α -SMA participated in isoproterenol-induced rat myocardial fibrosis with the kinetics similar to grade of fibrotic lesions. Additionally, the possible origin of the cells might be Thy-1-positive immature mesenchymal cells, because the appearance corresponded to that of vimentin- and α -SMA-positive cells. Furthermore, the formation of myocardial myofibroblasts might be regulated by increased level of TGF- β 1. These findings will provide fundamental information which may contribute partly to clarification of the pathogenesis of myocardial fibrosis.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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