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Original Article

Heart-derived fibroblasts express LYPD-1 and negatively regulate angiogenesis in rat

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ABSTRACT

Angiogenesis is regulated by a balance between promoting and inhibitory mechanisms. Although angiogenesis-promoting mechanisms have been well studied in ischemic heart diseases, angiogenesis-inhibitory mechanisms have not. Recently, we identified LYPD-1 as a novel anti-angiogenic factor derived from human heart-derived fibroblasts, which suppresses endothelial cell network formation in co-culture. However, it remains unclear whether the low angiogenicity of heart-derived fibroblasts with high expression of LYPD-1 is also observed in other mammalian species, and the properties of LYPD-1 under normal and pathological conditions remain elusive. Fibroblasts isolated from neonatal and adult rat heart also express LYPD-1 and inhibit endothelial network formation in co-culture. Moreover, immunohistochemical analysis revealed that LYPD-1 was predominantly observed in the interstitial tissues of rat heart and LYPD1 expression levels were identical from late developmental period to adult. Conversely, LYPD-1 mRNA expression was significantly downregulated temporally in myocardial infarction model rats, suggesting that angiogenesis-inhibitory mechanisms might not be sufficiently suppressed to promote angiogenesis in ischemic heart diseases. These findings suggest that heart has relatively low angiogenicity compared with other organs via the high expression of LYPD-1 by fibroblasts. Moreover, understanding the regulatory mechanisms of LYPD-1-mediated inhibition of angiogenesis might lead a novel angiogenic therapy for ischemic heart diseases and contribute to development of bioengineered cardiac tissue.
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1. Introduction

Angiogenesis is a biological process that is essential for tissue growth, homeostasis, and wound healing. Various angiogenic growth factors including VEGF promote angiogenesis upon injury, and such growth factor expression has been reported to be upregulated after myocardial infarction [1,2]. However, the endogenous upregulation of angiogenic growth factors is not sufficient to induce revascularization and therapeutic strategies without coronary intervention for the culprit lesion often lead to an increase of infarct size and heart failure. Therefore, major efforts have been made worldwide to develop angiogenic therapy and many researchers including us have reported that gene therapy targeting VEGF and cell therapy using various types of stem/progenitor cells enhance microvascular vessel generation and reduce the infarct size in animal myocardial infarction models and in a clinical setting [3–5]. However, angiogenesis is regulated by not only angiogenesis-promoting mechanisms, but also inhibitory ones. Angiogenesis-promoting factors promote the proliferation of endothelial cells by activating protein kinases such as Akt and Erk, and degradation of the extracellular matrix through matrix metalloproteinases generates a microenvironment suitable for endothelial cell sprouting [6,7]. In contrast, angiogenesis-inhibitory factors including endostatin, a fragment of collagen XVIII produced by proteolytic processing, inhibit endothelial cell proliferation,

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migration, and tube formation mainly by downregulating proangiogenic pathways [8,9]. The administration of endostatin has been reported to attenuate tumour growth in humans [10]. However, there are few reports about modification in angiogenesis-inhibitory mechanisms for the purpose of enriched blood perfusion that possibly leads recovery of organs with ischemia or fabrication of cardiac tissue.

Heart is composed of various types of cell, of which fibroblasts are the major component, constituting over half of all cardiac cells [11]. Although the principal reason for heart failure with systolic dysfunction is the loss of cardiomyocytes due to injury including myocardial infarction, cardiac fibroblasts have been reported to be responsible for the ensuing fibrosis and remodelling following myocardial infarction [12]. Understanding the underlying molecular mechanisms should lead to the development of new therapies for heart failure. However, our insufficient understanding of the principal phenotypes of heart-derived fibroblasts under physiological conditions makes it difficult to elucidate their function under pathological conditions. Recently, we identified a novel angiogenesis-inhibitory factor, LYPD-1, derived from human heart-derived fibroblasts, which suppresses endothelial cell network formation in co-culture. LYPD-1 is highly expressed in human heart-derived fibroblasts compared with its level in dermal tissue-derived fibroblasts and inhibition of LYPD-1 attenuates the inhibitory effects on angiogenesis mediated by human heart-derived fibroblasts [13]. Furthermore, recombinant LYPD-1 treatment suppresses dermal fibroblast-mediated endothelial cell network formation, suggesting that LYPD-1 has the ability to inhibit angiogenesis. Based on these findings, we hypothesize that heart might have relatively low angiogenicity compared with other organs through the high expression of LYPD-1 in fibroblasts. However, it remains unclear whether the low angiogenicity of heart-derived fibroblasts with high expression of LYPD-1 is preserved in other mammalian species. Furthermore, certain properties of LYPD-1 such as its localization in heart and its expression levels under normal and pathological conditions have been remained elusive.

The present study demonstrates that fibroblasts isolated from neonatal and adult rat hearts have anti-angiogenic properties through the high expression of LYPD-1. LYPD-1 is also highly expressed in heart from the embryonic period to adulthood and localized in the interstitial space of heart. However, LYPD-1 expression was not downregulated sufficiently in the myocardial infarction model. Taking these findings together, heart might normally be an organ with relatively low angiogenicity through the high expression of LYPD-1 in fibroblasts. Moreover, the more sufficient inhibition or the longer downregulation of LYPD-1 could lead to a novel angiogenic therapy for myocardial infarction.

2. Results

2.1. Heart-derived fibroblasts inhibit endothelial cell network formation

We first isolated heart-derived cells from adult rat hearts and dermal tissues in accordance with the fibroblast isolation methods described in previous reports. The cells isolated from each tissue showed typical spindle-shaped morphology (data not shown) and about 90% of the cells were positive for vimentin and CD90 (Fig. 1), suggesting that they were fibroblasts. Next, we co-cultured neonatal heart-derived CD31-positive endothelial cells with each type of fibroblast to evaluate the angiogenicity of these cells. Four days after starting co-culture, CD31-positive network formation was clearly observed upon co-culture with fibroblasts isolated from dermal tissues (Fig. 2A). In contrast, we observed the significant attenuation of endothelial network formation in co-culture with fibroblasts isolated from hearts (Fig. 2A), suggesting that the lower angiogenicity of heart-derived fibroblasts might also be preserved in rats. Next, we elucidated whether fibroblasts isolated from neonatal hearts also had less angiogenic potential because heart tissue grows in the neonatal stage and the angiogenic potential of fibroblasts might differ between neonatal and adult heart. The findings for the cells isolated from neonatal heart and dermal tissues also suggested that they were fibroblasts because almost all cells were positive for vimentin and CD90 (Fig. 1). When neonatal heart-derived CD31-positive cells were co-cultured with fibroblasts isolated from neonatal hearts, little endothelial network formation was observed, while co-cultivation with neonatal dermal fibroblasts significantly promoted endothelial cell network formation (Fig. 2B). These findings suggest that heart-derived fibroblasts might have less angiogenic potential regardless of the postnatal period.

2.2. LYPD-1 is the responsible factor for the low angiogenesis mediated by rat heart-derived fibroblasts

We recently identified that LYPD-1 is the factor responsible for the low angiogenesis associated with human heart-derived fibroblasts. Therefore, we compared the expression levels of LYPD-1 in fibroblasts isolated from heart and from dermal tissues. mRNA expression of LYPD-1 was hardly observed in fibroblasts isolated from neonatal and adult dermal tissues, while its abundant expression was observed in fibroblasts isolated from neonatal and adult hearts (Fig. 3A). Next, we elucidated whether the lower angiogenic potential of heart-derived fibroblasts is mediated by LYPD-1. When neonatal rat heart-derived CD31-positive endothelial cells were co-cultured with fibroblasts isolated from neonatal rat hearts in the presence of neutralizing antibody against LYPD-1, significant improvement of endothelial network formation was observed (Fig. 3B and C). These findings suggest that LYPD-1 might also be responsible for the low angiogenic potential of heart-derived fibroblasts, regardless of species.

2.3. LYPD-1 expression in normal rats

Although fibroblasts isolated from hearts highly expressed LYPD-1, it remained unclear whether heart tissues also express LYPD-1. As shown in Fig. 4A, mRNA expression levels of LYPD-1 were significantly high in neonatal rat hearts compared with those in neonatal dermal tissues and a similar difference in LYPD-1 expression was also observed between adult rat hearts and dermal tissues (Fig. 4A). There was no significant difference in LYPD-1 expression between neonatal heart tissues and adult heart tissues. Furthermore, mRNA expression levels of LYPD-1 were comparable in hearts from the embryonic stage (E15 and E18) to neonatal period (Fig. 4B). Next, we compared the expression of LYPD-1 among tissues/organs in normal adult rats. LYPD-1 expression was clearly observed in heart and brain, but not in testis, liver, kidney, spleen, lung, or oesophagus (Fig. 4C), suggesting that angiogenesis inhibitory machinery is more potent in heart in terms of high LYPD-1 expression.

Because of its high LYPD-1 expression in vitro, it remained unclear what types of cells express LYPD-1 in vivo. Immunohistochemical analysis revealed that LYPD-1 expression was observed in the interstitial space of heart (Fig. 4D), suggesting that fibroblasts might be the dominant cells expressing LYPD-1 in heart.

2.4. LYPD-1 expression in myocardial infarction model

In cases of myocardial ischemia, it is expected that endogenous tissue repair machinery that promotes angiogenesis is activated;

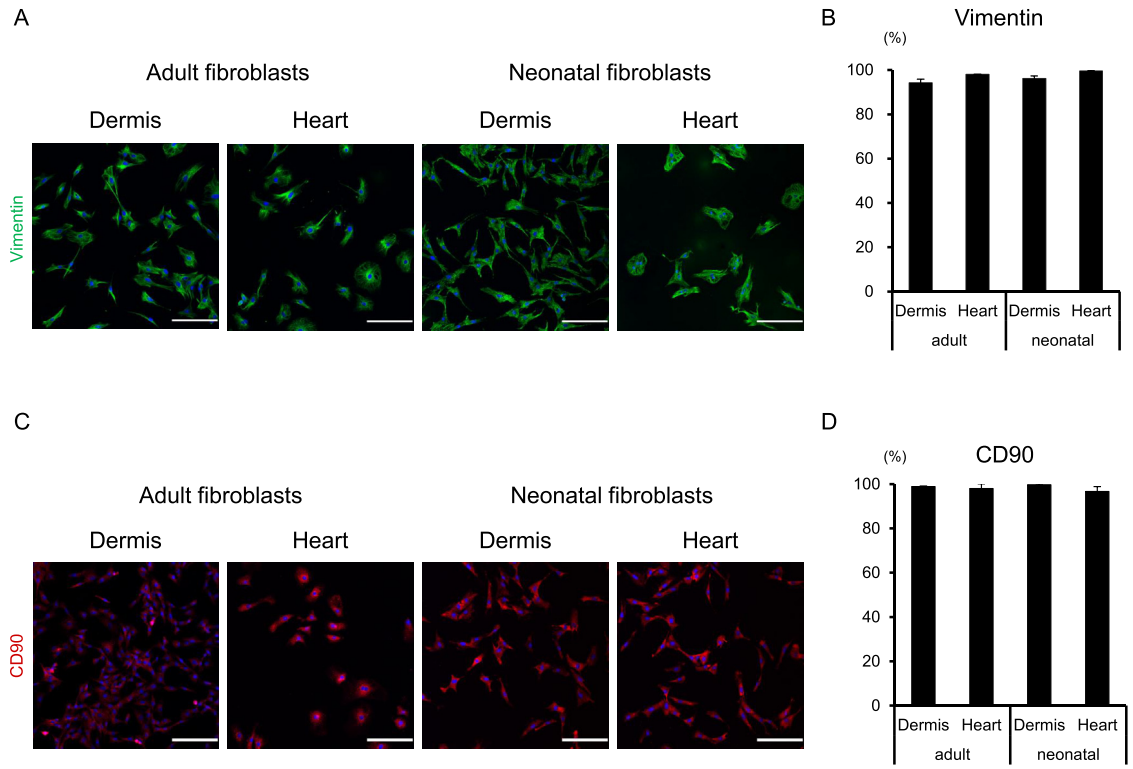

Fig. 1. The characteristics of fibroblasts. (A, C) Representative images of vimentin-positive cells (A) and CD90-positive cells (C) in each type of fibroblast. Bars, 200 μm. Nuclei were stained with Hoechst 33258. (B, D) The percentages of vimentin-positive cells (B) and CD90-positive cells (D) were calculated and are shown in the graph (n = 3).

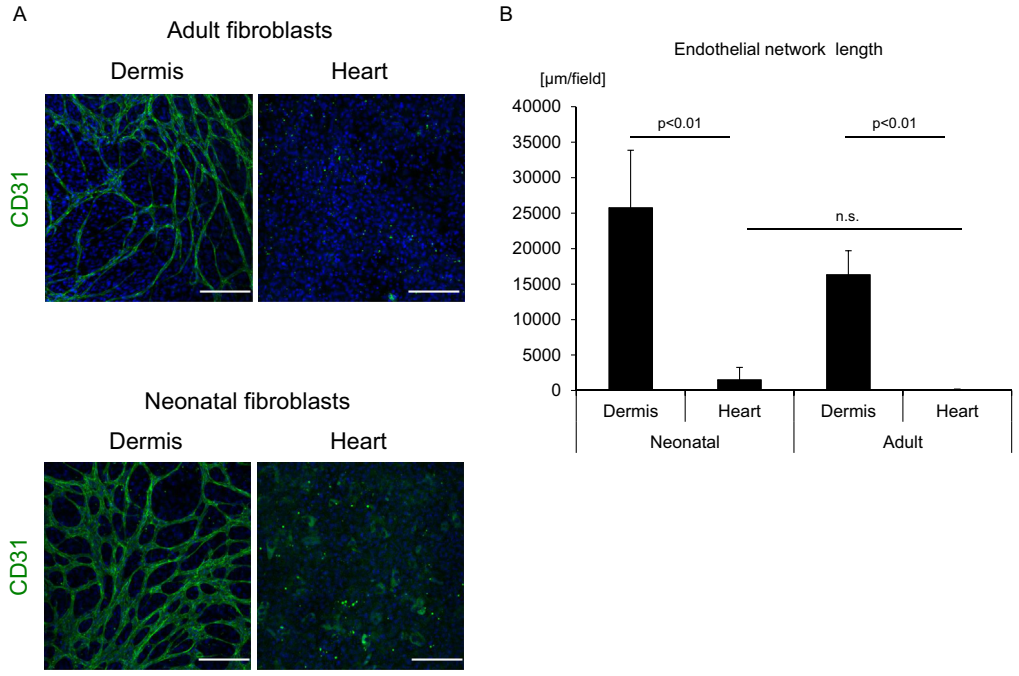

Fig. 2. Rat heart-derived fibroblasts inhibit endothelial network formation in co-culture. (A) The co-culture experiments of neonatal rat heart-derived CD31-positive endothelial cells with adult and neonatal fibroblasts isolated from dermal tissues and heart. (A) Representative images of CD31-positive endothelial network formation. Nuclei were stained with Hoechst 33258. Bars, 200 μm. (B) The length of endothelial network formation was calculated and is shown in the graph. Neonatal: p = 0.00003 (n = 4). Adult: p = 0.001 (n = 4).

moreover, proangiogenic growth factor expression has been reported to be upregulated in such cases [1,2]. However, it remains completely unclear how angiogenesis-inhibitory mechanisms are influenced by ischemia. Therefore, we generated a rat myocardial infarction model by permanently ligating the LAD and examined the expression levels of LYPD-1. Interestingly the LYPD-1

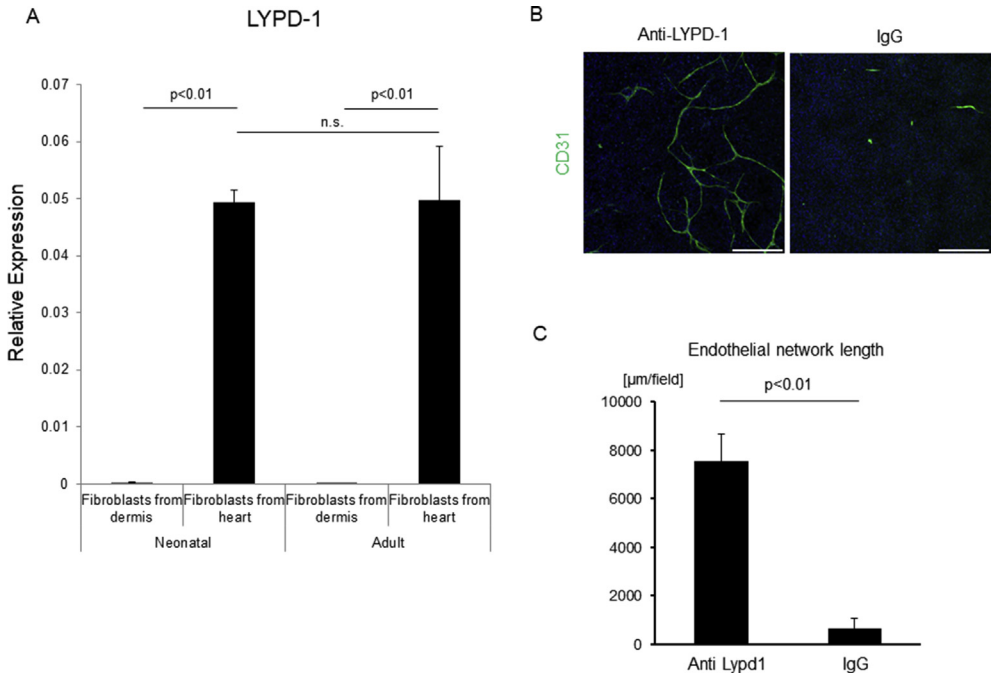

Fig. 3. LYPD-1 mediated inhibition of endothelial network formation in heart-derived fibroblasts. (A) qRT-PCR analysis of LYPD-1 expression in neonatal fibroblasts (left, n = 3, p = 0.000009) and adult fibroblasts (right, n = 3, p = 0.000009). (B, C) Recovery of endothelial network formation by neutralizing antibody against LYPD-1. (B) Representative images of CD31-positive endothelial network formation. (C) Total length was calculated from $CD31^+$ cell staining and is shown in the graph (n = 3, p = 0.002). Nuclei were stained with Hoechst 33258. Bars, 400 μm. Values are shown as mean ± standard deviation for three separate experiments.

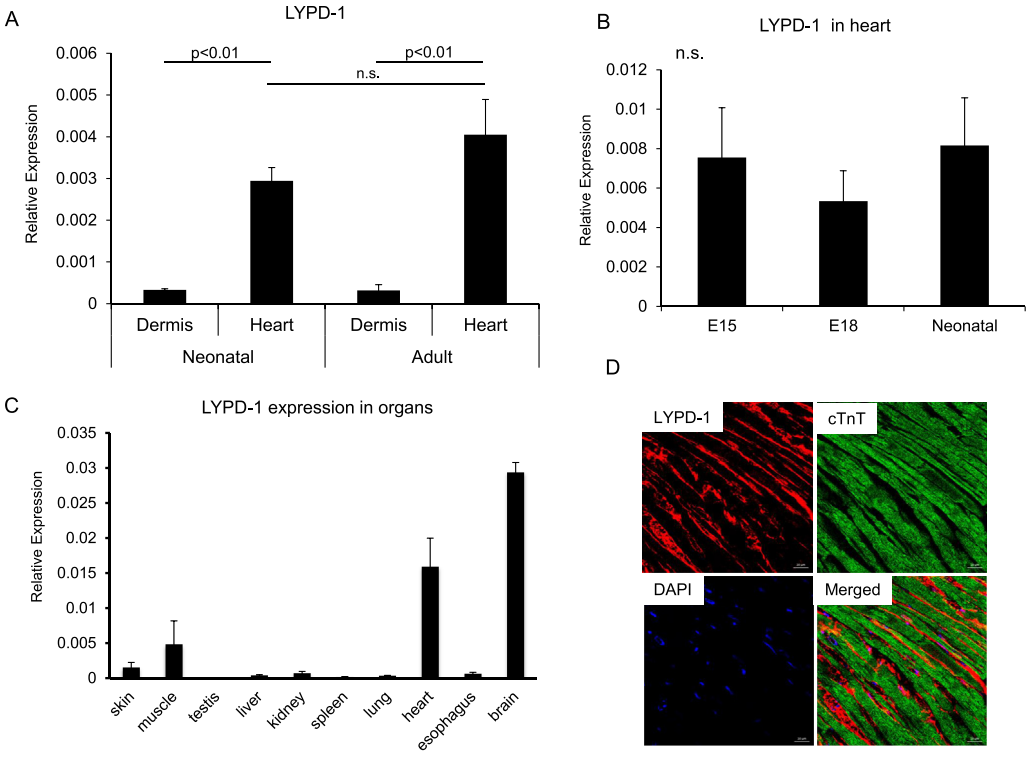

Fig. 4. LYPD-1 expression analysis in heart. (A) qRT-PCR analysis of LYPD-1 expression in neonatal dermis and heart (left, n = 4, p = 0.001) and adult dermis and heart (right, n = 4, p = 0.00004). (B) qRT-PCR analysis of LYPD-1 expression in rat hearts at various developmental stages (n = 4). n.s.: not significant. (C) qRT-PCR analysis of LYPD-1 expression in adult rat tissues and organs (n = 3, 8 weeks). (D) Immunohistochemical analysis of LYPD-1 in adult rat heart (8 weeks). Bars, 50 μm.

expression levels in hearts were significantly downregulated 1 day after myocardial infarction, but gradually recovered thereafter and reached the basal level 8 days after myocardial infarction (Fig. 5). These findings suggest that the lower angiogenicity mediated by LYPD-1 might be inhibited for quite a short period after myocardial infarction.

3. Discussion

In the present study, we showed that heart-derived fibroblasts from neonatal and adult rats inhibited endothelial network formation in co-culture conditions through their high expression of LYPD-1. LYPD-1 is specifically expressed in heart and brain, and the expression levels were found to be identical between the late stage of development and the neonatal period.

LYPD-1 expression was shown to be localized in the interstitial space of the heart. Furthermore, such mRNA expression was significantly and transiently downregulated after myocardial infarction, but then recovered to the basal level around 1 week after myocardial infarction.

Recently, we identified that LYPD-1 is a novel angiogenesis-inhibitory factor, by analysing human heart-derived fibroblasts [13]. LYPD-1 is a member of the lymphocyte antigen-6 (Ly6)/urokinase-type plasminogen activator receptor (uPAR) superfamily, characterized by a Ly6/uPAR domain containing 10 cysteines in 80 amino acids, arranged in a distinct disulphide-bridge pattern to create a three-fingered structural motif [14]. However, the mechanisms regulating the expression of LYPD-1 and its receptor in endothelial cells remain unclear. Consistent with the results obtained in human heart-derived fibroblasts, fibroblasts isolated from neonatal and adult rat hearts also inhibited endothelial network formation through LYPD-1, suggesting that the angiogenesis-inhibitory effects of LYPD-1 might also be preserved in nonhuman species including rat and that angiogenesis-inhibitory function through LYPD-1 might be a common phenotype of heart-derived fibroblasts regardless of species.

High expression levels of LYPD-1 were observed in heart from neonatal and adult rats. Interestingly, the expression levels of LYPD-1 mRNA in heart were identical from E15 to neonatal period. These findings suggest that angiogenesis-inhibitory machinery might always be active in heart. However, the generation of new blood vessels accompanies the development and growth of tissues and organs in embryos, while new blood vessel formation is dormant in adult healthy heart. The discrepancies between our results and the biological phenomena might be explained by the differences in the regulation of blood vessel formation between the embryonic stage and adulthood. Millauer et al. reported that endothelial cells in the brain in embryos and in the early postnatal period express abundant flk-1 (VEGF receptor 1), but the expression levels are drastically decreased in adults [15]. These findings indicate that the high expression levels of flk-1 in endothelial cells and the high affinity against VEGF might promote new blood vessel formation in embryos by overcoming the inhibitory effect of LYPD-1. Identification of the receptor for LYPD-1 in endothelial cells through our further studies will lead to understanding of the role of LYPD-1 in blood vessel formation in the developmental stage.

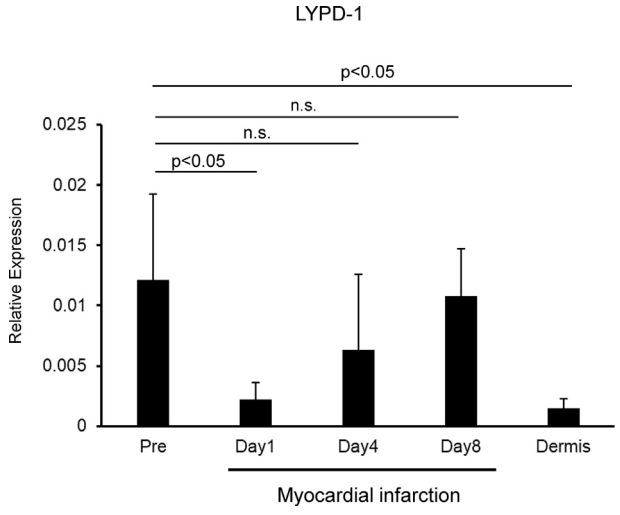

Fig. 5. LYPD-1 expression in the infarcted heart. qRT-PCR analysis of LYPD-1 mRNA expression in heart after myocardial infarction (n = 4, Day 1: p = 0.028, Dermis: 0.019). Dermis is shown as a reference of LYPD-1 low expression tissue.

As shown in Fig. 4C, LYPD-1 expression is specifically observed in heart and brain. Because vascular diseases of the heart and brain are leading causes of death in developed countries, the high expression of LYPD-1 seems to be paradoxical. However, the changes in the tissue environment during the process of angiogenesis might explain the meaning of the angiogenesis-inhibitory machinery in heart. Endothelial cell sprouting requires degradation of the extracellular matrix in tissues through the activation of several matrix metalloproteinases [7], suggesting that angiogenesis might accompany the structural reconstruction of tissues to a certain extent. The well-ordered alignment of cardiomyocytes and extracellular matrix-mediated tissue stiffness acting against high intraventricular pressure are imperative for heart pump function, the tissue reconstruction in accordance with angiogenesis might lead to the misalignment of cardiomyocytes and impaired tissue stiffness. Furthermore, the low rate of primary tumours in the heart [16] may support the lower angiogenicity of this organ through the high expression of LYPD-1.

An understanding of the role of angiogenesis-inhibitory mechanisms in ischemic heart should deepen our knowledge of the pathophysiology of ischemic heart diseases and could lead to the development of new methods that provides greater endothelial network in fabricating cardiac tissues. Moreover, the corresponding protein or agonists to LYPD1 receptor might have anti-angiogenic effect, which are potentially used as anti-tumour agent.

We observed the significant downregulation of LYPD-1 mRNA level 1 day after myocardial infarction, but that this recovered to the basal level at day 8, suggesting the insufficient responses to attenuate the angiogenesis-inhibitory mechanisms that hearts exhibit physiologically despite ischemia. The findings also suggest that the attenuation of LYPD-1 expression and function might be necessary for sufficient new blood vessel formation in myocardial infarction. Although the mechanism behind the transcriptional regulation of LYPD-1 is unclear, the evidence that LYPD-1 mRNA expression was downregulated 1 day after myocardial infarction might provide us with some clues to understand it. As LYPD-1 expression was localized in the interstitial spaces of normal heart, resident fibroblasts in normal heart are supposed to express LYPD1. On the other hand, bone marrow-derived fibroblasts have been reported to be present in fibrotic heart after myocardial infarction [17]. We previously reported the low expression levels of LYPD-1 in human bone marrow-derived mesenchymal stem cells [13]. The environmental cues in the heart including mechanical stress may activate the transcriptional regulation of LYPD-1 in the migrated fibroblasts to the level of resident fibroblasts. Since many inflammatory cells are well known to infiltrate to heart in the infarcted heart, that phenomenon might affect the LYPD-1 expression levels 1 day after MI. Further experiments will be necessary to clarify the mechanisms of LYPD-1 transient downregulation after myocardial infarction.

Recently, Fu et al. have reported about the new differentiated phenotype of resident cardiac fibroblasts after myocardial infarction [18]. In that report, they suggest that some resident cardiac fibroblasts were differentiated into activated cardiac fibroblasts 2–3 days after myocardial infarction, and were further differentiated into matrifibroblasts at 4–7 days after myocardial infarction. In the present study, LYPD1 expression levels in infarcted heart were recovered to those in non-infarcted heart at day 8. The drastic phenotypical changes of resident cardiac fibroblasts to myofibroblasts and matrifibroblasts might affect the LYPD1 expression levels in infarcted heart and the environmental cues to the phenotypical changes of resident fibroblasts might be critical for the transcriptional regulation of LYPD1.

Herein, we have shown the contribution of angiogenesis-inhibitory mechanisms in heart via LYPD-1 expression. Further understanding of the regulatory mechanisms of LYPD-1-mediated angiogenesis inhibition and the development of a strategy to inhibit LYPD-1 expression or function might provide us a novel angiogenic therapy for ischemic heart diseases and a strategy of engraftable cardiac tissue development.

4. Materials and methods

4.1. Animals and reagents

Wild-type Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan).

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University. The rats were anesthetized using 3%–5% sevoflurane, in compliance with ARRIVE guidelines [19].

The following antibodies were used for immunocytochemistry and magnetic-activated cell sorting (MACS): anti-rat CD31 (clone TLD-3A12; Bio-Rad, Hercules, CA), anti-cardiac Troponin T (#MA5-12960, Thermo Fisher Scientific, Waltham, MA), and anti-Vimentin mouse monoclonal antibodies (#Ab-8069, Abcam, Cambridge, UK), and anti-CD90 (#Ab-226, Abcam), Hoechst33258 (#H3569, Thermo Fisher Scientific) and anti-Lypd-1 (#Ab-157516, Abcam) rabbit polyclonal antibodies. Alexa Fluor 488 secondary antibody anti-mouse IgG was purchased from Thermo Fisher Scientific and all other secondary antibodies were purchased from Jackson Immuno-Research Laboratories (West Grove, PA). The MACS system including a magnet column and magnet beads were purchased from Miltenyi Biotec Inc. (Bergisch Gladbach, Germany). Collagenase type 2 was purchased from Worthington Biochemical (Lakewood, NJ).

4.2. Cell culture

Cells were cultured in Dulbecco's modified Eagle's medium (#D6429, Sigma-Aldrich, MO) supplemented with 10% foetal bovine serum (Japan Bio Serum, Tokyo, JAPAN) and 1% penicillin–streptomycin (Thermo Fisher Scientific) in humid air with 5% CO_2, unless otherwise indicated.

4.3. Isolation of neonatal rat heart-derived CD31-positive cells

Neonatal rat heart-derived CD31-positive cells were isolated as described previously [20]. Briefly, hearts from neonatal rats (1–3 days old) were minced into cubes with sides of approximately 1 mm^2, and dissociated with collagenase type 2 into a single-cell suspension. CD31-positive cells were isolated from the cell suspension using an endothelial cell isolation kit (Miltenyi Biotec Inc.), in accordance with the supplier's instructions.

4.4. Isolation and culture of neonatal rat heart-derived fibroblasts

Neonatal rat heart-derived fibroblasts were isolated by a slightly modified version of a previously described method [21]. After the dissociation of neonatal rat hearts and CD31 isolation as mentioned above, CD31-negative cells were cultured on uncoated culture plates in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin in humid air with 5% CO_2 for 1 h. Then, non-adherent cells were discarded and adherent cells were subsequently cultured as fibroblasts. Fibroblasts were passaged every 6 days and fibroblasts from passages 2–6 were used for the experiments.

4.5. Isolation and culture of adult rat heart-derived fibroblasts

Fibroblasts were isolated and cultured from the hearts of adult male wild-type rats (8–10 weeks old) using simplified and Langendorff-free procedures, as described elsewhere [22]. Fibroblasts were passaged every 6 days and fibroblasts from passages 2–6 were used for the experiments.

4.6. Isolation and culture of neonatal and adult rat dermal tissue-derived fibroblasts

Fibroblasts from the dermis of neonatal (1–3 days old) and adult (8–10 weeks old) rats were isolated using explant methods. The dermis was cut into cubes with sides of approximately 2 mm, which were then cultured on cell culture dishes. Six days after starting the culture of neonatal dermal tissues, cells grown on the dishes were isolated and cultured as neonatal fibroblasts. Seven to ten days after starting the culture of adult dermal tissues, cells grown on the dishes were isolated and cultured as adult fibroblasts. Fibroblasts were passaged every 4 days and fibroblasts from passages 2–6 were used for the experiments.

4.7. Co-culture of CD31-positive endothelial cells with fibroblasts

Neonatal rat heart-derived CD31-positive cells (2.4×10^5 cells/well) were co-cultured with each type of fibroblast (4×10^5 cells/well) on a multiwell plate (24-well; Corning Inc. Corning, NY). The medium was changed at day 2 and cells were fixed with 4% paraformaldehyde (Muto Pure Chemicals Inc., Japan) at day 4. In some co-culture experiments, anti-LYPD-1 antibody (5 μg/ml) and rabbit IgG (5 μg/ml) were added to the culture medium for 4 days.

4.8. Immunocytochemistry and immunohistochemical analysis

Fibroblasts were seeded in a 24-well culture plate (Corning) at a density of $1–4 \times 10^4$ cells/well and cultured for 1–2 days. The cells were then fixed with 4% paraformaldehyde and subjected to immunocytochemistry, as described previously [13]. Samples were imaged by ImageXpress (Molecular Device, Sunnyvale, CA) with MetaXpress and AcuityXpress software (Molecular Device).

Hearts from adult rats were quickly frozen in liquid nitrogen. Six-millimetre frozen sections were then prepared and fixed with 4% paraformaldehyde. Samples were preblocked, and stained with primary and secondary antibodies as described previously [13]. Each antibody was diluted with a staining solution (1:100). Images were taken under a laser confocal microscope (Carl Zeiss, Germany).

4.9. RNA extraction and quantitative reverse-transcription PCR

Total RNA from isolated fibroblasts was extracted using RNeasy Plus Mini Kit and QIA Shredder Kit (QIAGEN, the Netherlands). Total

RNA from tissues was extracted by an acid guanidinium thiocyanate–phenol–chloroform extraction method using RNA BEE (Takara Bio Inc., Tokyo, Japan). RT-PCR was carried out using a One Step Prime-Script RT-PCR Kit (Takara Bio Inc.) on a STEP ONE PLUS PCR System (Takara Bio Inc.) and High-Capacity CDNA Reverse Transcription Kit (Thermo Fisher Scientific). TaqMan probes for rat LYPD1 (assay ID: Rn01753748_m1) and rat β-actin (ACTB) (assay ID: Rn00667869_m1) and were purchased from Thermo Fisher Scientific. Relative mRNA expression levels were calculated using a standard curve of β-actin mRNA levels.

4.10. Acute myocardial infarction model

Myocardial infarction model rats were created by permanent ligation of the left anterior descending coronary artery (LAD), in accordance with our previously described procedure [23]. Male adult rats (10 weeks old) were anesthetized using 3.5%–4.5% sevoflurane and were ventilated by a respirator. Hair on the chest was removed by clippers and then the chest was sterilized using 70% ethanol. After stable anaesthesia had been established, the chest was opened by left-sided thoracotomy and the beating heart was exposed. Next, 7-0 prolene (Ethicon, Somerville, NJ) was used to permanently ligate the LAD, after which 5-0 Vicryl (Ethicon) and 4-0 Nylon (Ethicon) were used to close the chest. Samples for quantitative RT-PCR were dissected from infarct area of the hearts and are quickly frozen in liquid nitrogen.

5. Statistical analysis

Data are presented as mean ± standard deviation. Welch's test was applied to compare two groups. Multiple group comparisons were performed by one-way analysis of variance followed by Tukey-Kremer method or Dunnett's procedures for comparison of means. Values of $p < 0.05$ were considered to be statistically significant. Statcel3 software and EXCEL Toukei ver. 7.0 (ESUMI Co., Ltd, Tokyo, Japan) were used for the data analysis.

Author contributions

KM designed the experiments. SS, KM and SM performed the experiments. NH and TS supervised the experiments. SS and KM wrote the manuscript. All authors read and approved the final manuscript.

Competing financial interest

There are no potential competing interests.

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