Overexpression of collagen type III in injured myocardium prevents cardiac systolic dysfunction by changing the balance of collagen distribution

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ABSTRACT

Objective: Left ventricular (LV) remodeling alters the contractile and relaxation properties and induces myocardial stiffness. As LV remodeling progresses, the amount of collagen type III (Col3) is gradually decreased, being replaced by collagen type I (Col1). We evaluated whether Col3 overexpression improved cardiac function and remodeling in a rat with ischemic cardiomyopathy (ICM). We also investigated the functional motif and mechanism of thrombin-cleaved N-terminal osteopontin (N-OPN) on cardiac remodeling.

Methods: The rats with ICM were divided into 3 groups: ligation only (Control) group and groups transplanted with nontransfected fibroblast sheets (normal Fb group) or with Col3-secretory fibroblast sheets (Col3 Fb group). A gelatin hydrogel containing the N-terminal fragment (N-OPN), N-OPN lacking the SVVYGLR sequence (Δ SV), the Arg-Gly-Asp (RGD) sequence (Δ RGD), RGD and SVVYGLR sequences (Δ RGD-SV), SVVYGLR alone (SV), or a random SV peptide was implanted into an ICM model rat.

Results: The Col3 Fb group exhibited significantly attenuated LV systolic dysfunction. LV dilatation, myocyte hypertrophy, and LV fibrosis at the infarcted area were also attenuated by Col3 Fb implantation. Furthermore, N-OPN, Δ RGD, and SV peptide suppressed the depression of cardiac function, LV dilatation, and myocyte hypertrophy, and also induced increased Col3 expression and reduction in the ratio of Col1 to Col3 in the infarcted and border areas.

Conclusions: Overexpression of Col3 improved cardiac function by changing the balance of collagen distribution in LV remodeling. The SVVYGLR motif of the thrombin-cleaved N-OPN and SV peptide attenuated cardiac dysfunction by increasing Col3 and changing the pattern of collagen balance in the impaired area. (J Thorac Cardiovasc Surg 2018;156:217-26)

Cardiac systolic function after implantation of Col3-secretory cell sheets.

Central Message

Increased collagen type III expression in injured myocardium maintains cardiac systolic function.

Perspective

Col3 is the collagen fiber laid down in cardiac injury. It is then replaced by Col1, which consequently leads to increased stiffness of the left ventricular (LV) wall and LV systolic dysfunction. We found that increasing Col3 distribution attenuated cardiac systolic dysfunction by changing the balance of the collagen ratio in LV remodeling. This finding provides a novel strategy for cardiac therapeutics.

See Editorial Commentary page 227.

Following myocardial infarction (MI), cardiomyocytes are triggered to undergo apoptosis, with the apoptotic region being replaced by collagen fiber along with a gradual loss of elastic fiber. The fibrotic left ventricular (LV) wall is intolerant of intracardiac pressure; consequently, LV

remodeling, such as expansion of the infarcted area, ventricular dilatation, myocyte hypertrophy, and thinning of the ventricular wall, ensues.¹⁻³ Extracellular matrix (ECM) deposition and organization play major roles in LV remodeling, with progressive LV remodeling

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Abbreviations and Acronyms

Col1	= collagen type I
Col3	= collagen type III
COL3a1	= collagen type III α 1 chain
ECM	= extracellular matrix
EF	= ejection fraction
Fb	= fibroblast
FS	= fractional shortening
ICM	= ischemic cardiomyopathy
LV	= left ventricular
MI	= myocardial infarction
N-OPN	= N-terminal osteopontin
OPN	= osteopontin
RGD	= Arg-Gly-Asp
SMA	= smooth muscle actin

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associated with increased interstitial collagen fiber a major cause of diastolic heart failure after MI.⁴⁻⁶ Notably, the ratio of collagen type I (Col1) and Col3 within cardiac interstitial tissue has an influence on both cardiac diastolic and systolic function. In the LV remodeling process, the level of Col1 is increased whereas that of Col3 is decreased.^{4,7,8} In particular, this increase in the ratio of Col1 to Col3 contributes to reduced elasticity of the LV wall and LV dilation,^{9,10} because Col3, whose helical molecules form a homotrimer of α 1 chains,¹¹ is an important component for maintaining the normal morphological structure and physiological function of cardiac tissue.⁴

Osteopontin (OPN) is an acidic phosphorylated glycoprotein containing an integrin-binding Arg-Gly-Asp (RGD) sequence.¹² OPN biological functions, such as bone remodeling, macrophage response, cell migration, and adhesion, are modulated by thrombin cleavage generating N- and C-terminal fragments.¹³⁻¹⁵ Thrombin cleavage of OPN is required for integrin $\alpha_9\beta_1$ interaction with the resulting N-terminal fragment via the motif ¹⁶²SVVYGLR¹⁶⁸.^{16,17} In previous studies, we found that the SVVYGLR (SV) motif of the N fragment induced the activation of Smad signaling, leading to α -smooth muscle actin (SMA) expression in cardiac fibroblasts.^{18,19} The SV peptide and OPN thrombin-cleaved N fragment increased the Col3 expression level in cardiac fibrosis and maintained cardiac function in a rat after ischemic cardiomyopathy (ICM) and in a dilated

cardiomyopathy model hamster.^{18,20} However, this effect was evaluated over a short duration (2 weeks), and the influence of the RGD motif was not examined. In addition, the effects of increased Col3 expression mediated by this fragment and of the peptide on cardiac function have not yet been fully evaluated.

Accordingly, the aim of the present study was to examine the effects of Col3 expression on cardiac function. We transplanted fibroblast sheets that secrete Col3 into a model rat with ICM. Moreover, to identify the functional motif and mechanism of thrombin-cleaved N-terminal osteopontin (N-OPN), we investigated the impact of N-OPN fragments on cardiac function and remodeling, focusing on collagen distribution.

METHODS**Ethical Approval**

All animal work was approved by the Ethical Review Committee of Osaka University and conducted in accordance with the National Institutes of Health's (NIH) Animal Protocol Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 2011).

Isolation and Culture of Primary Cardiac Fibroblasts

After excision from male 3-week-old Lewis rats (Japan SLC, Shizuoka, Japan) under an overdose of sodium pentobarbital sedation (50 mg/kg, intraperitoneal injection) and heparin (150 U), the removed heart was separated from the atria, and the ventricular tissue was minced and washed with Krebs-Henseleit buffer solution. Primary adult rat ventricular fibroblasts (Fbs) were isolated using 0.2% collagenase type II (Worthington Biochemical, Lakewood, NJ) and 0.1% trypsin. After centrifugation, the pelleted cells were suspended in Dulbecco's modified Eagle's medium (Nihon-seiyaku, Tokyo, Japan) containing 10% fetal bovine serum.

Construction of a Human Col3-Expressing Plasmid

A fragment of human Col3 α 1 chain (COL3a1) cDNA including a HindIII site and secretory signal sequence at the 5' end and XbaI sites at the 3' end (Figure 1, A and Figure E1) was synthesized by GenScript (Piscataway, NJ). The synthesized human COL3a1 cDNA fragment was inserted into between the HindIII and XbaI sites of the pcDNA3.1 vector.

Preparation of Human COL3a1-Overexpressing Fbs

The pcDNA3.1 expression plasmid encoding a fragment of human Col3a1 was transfected into the isolated primary Fbs using Lipofectamine (Invitrogen Life Technologies, Waltham, Mass). Stable clones expressing human Col3a1 were selected by culture under the presence of the antibiotic neomycin.

Western Blot Analysis

Cellular media were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred and incubated with anti-human Col3 polyclonal antibody (Protein-tech Group, Chicago, Ill). The bands were visualized using an ECL kit. In contrast to the control vector cells (mock) lacking human COL3A1 cDNA, Col3 was not secreted (Figure 1, B).

Transplantation of Human COL3a1-Overexpressing Fb Sheets

To generate ICM model rats, 8-week-old male Lewis rats (Japan SLC) were anesthetized using inhaled isoflurane (2%; 0.2 mL/min) and

FIGURE 1. The preparation of Col3-secretory fibroblast sheets and recombinant thrombin-cleaved N-terminal osteopontin (N-OPN) fragments. **A**, View showing the frame format of the constructed *COL3A1* gene. **B**, Assessment of expression and secretion of the recombinant Col3 from fibroblasts by Western blot analysis. **C**, Schematic view of the produced recombinant thrombin-cleaved N-OPN fragments. *RGD*, Arg-Gly-Asp.

intubated. MI was induced in the rats by ligation of the left anterior descending coronary artery (LAD). At 2 weeks after LAD ligation, Fb sheets were implanted on the infarcted LV anterior wall of the 10-week-old Lewis rats. The ICM model rats were divided into 3 treatment groups: Col3 Fb group (implantation of 3 Col3-secreting Fb sheets; $n = 7$), (2) normal Fb group (implantation of 3 nontransfected Fb sheets; $n = 7$), and (3) control group (ligation only; $n = 7$). The 3 fibroblast sheets were sequentially applied to the surface of the infarcted myocardium. After confirming the adherence of the first sheet, subsequent sheets were applied using the same technique, as described previously.²¹⁻²³

Production of Human Recombinant OPN Proteins

The coding sequences lacking the signal sequence of the thrombin-cleaved OPN fragments (N-OPN; amino acids 17–168), SVVYGLR sequence-removed N-terminal OPN (Δ SV; amino acids 17–161), RGD sequence-removed N-terminal OPN (Δ RGD; amino acids 17–158 and 162–168), and both SVVYGLR and RGD sequence-removed N-OPN (Δ RGD-SV; amino acids 17–158) were amplified by polymerase chain reaction with specific oligonucleotides and primers and cloned into the pGEX-4T-2 vector (Figure 1, C). The expression vectors were transformed into *Escherichia coli* BL21, and the expressed recombinant OPN fragments were purified as described previously.⁷

Recombinant OPN Fragment or SV Peptide Sustained-Release Collagen Gel Treatment

ICM model rats were generated as described previously using 8-week-old male Sprague–Dawley rats (Charles River Laboratories Japan, Yokohama, Japan). At 2 weeks after LAD ligation, a biodegradable gelatin hydrogel (MedGel; MedGEL Corporation, Tokyo, Japan) was implanted on the LV anterior wall of the ICM model rat, as described previously.^{18,20} The gel incorporated a 1/10 phosphate-buffered saline (PBS) solution containing each recombinant OPN fragment, SV peptide, or random SV peptide (GYRVLSV) (each 50 μ g/gel). The implanted MedGel was slowly resolved by collagenase *in vivo*, allowing the gradual administration of physiologically active OPN fragments over a long period. A previous report confirmed that the substance integrated into the gel exhibited sustained release for 14 days after implantation *in vivo*.¹⁸ The rats were divided into 6 groups: N-OPN, Δ SV, Δ RGD, Δ RGD-SV, SV peptide (SV), and random SV peptide (random). As a control, a peptide-free gel containing only PBS solution was implanted similarly ($n = 5$ each group).

Measurement of Cardiac Function by Echocardiography

The cardiac function of the treated rats was monitored by echocardiography both before surgery and at 2, 4, 6, and 8 weeks after gel implantation or at 2 and 4 weeks after cell sheet implantation using a system equipped with a 12-MHz transducer and a SONOS 5500 device (Agilent Technologies, Santa Clara, Calif) under isoflurane inhalation (2%), as described previously.^{19,22,23}

Histological Analysis

At 4 or 8 weeks after treatment, the excised and fixed LV myocardium specimens were processed for histology as described previously^{22,23} (also see Supplemental Methods).

Statistical and Power Analysis

Data are expressed as mean $\pm$ SD. Differences among groups of rats at the endpoint were assessed by 1-way factorial analysis of variance; if a significant difference was detected, intergroup comparisons were performed with Tukey's honest significant difference test. Time courses of cardiac functional parameters were compared among groups by 2-way repeated-measures analysis of variance. A P value $< .05$ (2-sided) was considered statistically significant. We performed post hoc power analysis using G*Power software.

RESULTS

Effect of Col3 Expression on Cardiac Function

No major differences at baseline were observed among the 3 experimental groups (control vs normal Fb, $P = .96$; control vs Col3 Fb, $P = .23$; normal Fb vs Col3 Fb, $P = .79$). The Col3 Fb group exhibited significant improvement in the percentage of fractional shortening (%FS) and ejection fraction (EF) at 2 weeks ($P = .003$) and 4 weeks ($P < .001$) compared with the control group. Furthermore, at 4 weeks, significant improvements in %FS ($P = .02$) and EF ($P = .02$) was detected between the Col3 Fb and normal Fb groups (Figure 2, A and B), although the changes in percentage were small.

FIGURE 2. Time course of changes in left ventricular systolic function obtained by echocardiography over the 4-week period after sheet transplantation. A, Fractional shortening ($n = 7$). B, Ejection fraction ($n = 7$). * $P < .05$ versus control, # $P < .05$ versus normal. FS, Fractional shortening; Fb, fibroblast; EF, ejection fraction.

In contrast, there was no significant difference in both LV end-diastolic and systolic diameter (LVDD and LVDs) among the 3 experimental groups at all measurement points (Table E1).

Effect of Col3 Expression on LV Remodeling

At 4 weeks after sheet implantation, the control and normal Fb groups showed similar morphological changes, such as LV ventricular dilation ($P = .25$), cardiac remodeling ($P = .45$), and cardiomyocyte hypertrophy ($P = .25$) (Figure 3, A-F). However, the Col3 Fb group showed significantly attenuated LV ventricular dilation ($P = .03$; Figure 3, A and B). Significant reductions in fibrosis (vs control, $P < .001$; vs normal Fb, $P < .001$; Figure 3, C and D) and cardiomyocyte size (vs control, $P < .001$; vs normal Fb, $P < .001$; Figure 3, E and F) at infarcted border areas was observed in the Col3 Fb group as well.

Distribution of Collagen Fiber Following Col3 Fb Sheet Implantation

Immunohistological staining showed no significant differences in the distribution level of Col1 at infarcted areas among the 3 experimental groups (control vs normal Fb, $P = .48$; control vs Col3 Fb, $P = .65$; normal Fb vs Col3 Fb, $P = .78$; Figure 3, G and I). Conversely, Col3 expression levels were significantly higher at the infarcted area in the Col3 Fb group compared with the control and normal Fb groups (vs control, $P < .001$; vs normal Fb, $P < .001$, Figure 3, H and J). The ratio of Col1 to Col3 was significantly lower in the Col3 Fb group compared with the control and normal Fb groups (vs control, $P < .001$; vs normal Fb, $P < .001$; Figure 3, K).

Evaluation of Cardiac Function Following Gel Treatment

Systolic function was markedly decreased over time in the control, Δ SV, Δ RGD-SV, and random groups, whereas the N-OPN ($P < .001$), Δ RGD ($P < .001$), and SV groups ($P < .001$) exhibited significant suppression of this dysfunction at 8 weeks compared with the control group. This inhibition effect was similar among the 3 groups (EF: N-OPN vs Δ RGD, $P = .49$; N-OPN vs SV, $P = .64$; Δ RGD vs SV, $P = .22$; FS: N-OPN vs Δ RGD, $P = .68$; N-OPN vs SV, $P = .66$; Δ RGD vs SV, $P = .37$; Figure 4, A and B). In contrast, both LVDD and LVDs were not significantly different among all experimental groups (Table E2).

Effects of Thrombin-Cleaved OPN N-Fragment on LV Dilation and Cardiac Hypertrophy

Histological analysis revealed that at 8 weeks after gel implantation, LV dilation (Figure 5, A and B) and the LV cardiomyocyte cross-sectional size (Figure 5, C and D) were expanded in the control, Δ SV, Δ RGD-SV, and random groups. Conversely, these changes were significantly attenuated in the N-OPN group (LV dilation, $P = .04$; cardiomyocyte size, $P < .001$), Δ RGD (LV dilation, $P = .005$; cardiomyocyte size, $P < .001$), and SV group (LV dilation, $P = .03$, cardiomyocyte size, $P < .001$) compared with the control group.

Distribution of SMA and Collagen Fiber and After Gel Implantation

The degree of myofibroblast accumulation assessed by immunostaining for α -SMA in both the infarcted area (Figure 5, E and F) and the border area (Figure 5, G

FIGURE 3. Histological evaluation of left ventricular (LV) tissue to determine LV dilation, cardiomyocyte hypertrophy, and fibrosis after 4 weeks of sheet treatment. A, Hematoxylin and eosin–stained images. (Scale bars: 1000 μ m.) B, Ratio of LV diameter to interventricular septal thickness (n = 7). C and E, Representative photomicrographs of LV cross-sections at the infarcted border area stained with Sirius red (C) and periodic acid Schiff (E). D and F, Quantification of cardiomyocyte size and fibrosis (n = 7). G and H, Immunohistochemical analyses of 4-week rat hearts of ischemic cardiomyopathy for Col1 (G) and Col3 (H) at the infarcted area. I and J, Percentage of distribution of Col1 (I; n = 7) and Col3 (J; n = 7). K, Ratio of Col1 to Col3 (n = 7). ** P < .01, * P < .05 versus control; ## P < .01 versus normal. Fb, Fibroblast; Col3, collagen type III; LV, left ventricular; Col1, collagen type I.

FIGURE 4. Echocardiography assessment of fractional shortening (A) and ejection fraction (B) as measured before and 2, 4, 6, and 8 weeks after gel implantation to assess left ventricular function. $n = 5$ in each group. $^{**}P < .01$ versus control. FS, Fractional shortening; N-OPN, N-terminal osteopontin; SV, SVVYGLR sequence; RGD, Arg-Gly-Asp; EF, ejection fraction.

and H). It was significantly higher in rats treated with recombinant N-OPN (infarcted and border areas, $P < .001$), Δ RGD (infarcted and border areas, $P < .001$), and SV peptide (infarcted and border areas, $P < .001$) compared with the control group. The Δ SV (infarcted, $P = .003$; border, $P = .03$) and Δ RGD-SV (infarcted; $P = .02$) groups also exhibited increased SMA expression in the infarcted and/or border areas, albeit to a lesser degree.

The distribution of Col1 in the gel-implanted infarcted area was similar in all experimental groups ($P = .37-.52$; Figure 6, A and B). Conversely, the amount of Col3 expression in the N-OPN ($P < .001$), Δ RGD ($P < .001$), and SV ($P < .001$) groups was greater than that in the control group (Figure 6, C and D). In the infarcted border area, the degree of Col1 expression in the N-OPN ($P < .001$), Δ RGD ($P < .001$), and SV ($P < .001$) groups was significantly decreased (Figure 6, E and F), whereas that of Col3 in these 3 groups was increased relative to the control group (vs N-OPN, $P < .001$; vs Δ RGD; $P < .001$; vs SV, $P < .001$; Figure 6, G and H). Consequently, the ratio of Col1 to Col3 in both the infarcted area (Figure 6, I) and border area (Figure 6, J) was also reduced in the N-OPN (infarcted and border areas, $P < .001$), Δ RGD (infarcted and border areas, $P < .001$), and SV (infarcted and border areas, $P < .001$) groups. The Col1 distribution level in the infarcted border area, and that of Col3 and the Col1/Col3 ratio in both the infarcted and border areas in the Δ SV (distribution of Col1, $P = .16$; distribution of Col3: infarcted, $P = .59$; border, $P = .16$; Col1/Col3 ratio: infarcted, $P = .58$; border, $P = .36$), Δ RGD-SV (distribution of Col1, $P = .78$; distribution of Col3: infarcted, $P = .65$; border, $P = .11$; Col1/Col3 ratio: infarcted, $P = .79$; border,

$P = .47$), and random (distribution of Col1, $P = .95$; distribution of Col3: infarcted, $P = .79$; border, $P = .58$; Col1/Col3 ratio: infarcted, $P = .72$; border, $P = .66$) groups were comparable to those of the control group.

DISCUSSION

In this study, we have shown that (1) overexpression of Col3 attenuated cardiac systolic dysfunction by changing the balance of collagen distribution and ratio in LV remodeling; (2) the SVVYGLR motif induced Col3 expression levels in the injured myocardium; and (3) the SVVYGLR motif, but not the RGD motif, in N-OPN plays a main role in the suppression of cardiac function disruption and LV remodeling by increasing Col3 and changing the pattern of collagen balance. In the vascular structure, Col1 confers rigidity, whereas Col3 promotes vascular elasticity.²⁴ Specifically, higher levels of Col3 provide more elasticity to the ECM; for example, the stiffness of the aorta has been related to relative Col1 and Col3 abundance.²⁵ Moreover, it has been reported that increased myocardial levels of Col1 cause unsuitable remodeling and deterioration of cardiac function owing to pressure overload, whereas increased levels of Col3 are associated with myocardial distensibility, leading to improved cardiac function.²⁶ Col3 composes the initial collagen laid down on cardiac injury and is usually replaced by Col1, which consequently leads to increased stiffness of the LV wall and LV systolic dysfunction.^{8,9,27} Consistent with these findings, in the present study, the implantation of Col3-secreting Fbs sheets suppressed the reduction of cardiac systolic function after ischemic damage, demonstrating that the increased level of Col3 distribution is an important factor in maintaining

FIGURE 5. Histological changes of injured myocardium at 8 weeks after gel implantation. A, Representative image of heart sections stained with hematoxylin and eosin. (Scale bars: 1000 μm .) B, Quantification of the left ventricular diameter to interventricular septal thickness ratio. C, Representative images of periodic acid Schiff-stained heart sections. D, Quantification of cardiomyocyte size in the infarcted area. E and G, Immunostaining with anti- α -SMA antibody in the infarcted (E) and border (G) areas performed on myocardium cross-sections. F and H, Percentage of SMA expression level per field area determined from sections similar to those in (E) and (G). $n = 5$ in each group. $**P < .01$, $*P < .05$ versus control. N-OPN, N-terminal osteopontin; SV, SVVYGLR sequence; RGD, Arg-Gly-Asp; LV, left ventricular; SMA, smooth muscle actin.

FIGURE 6. Distribution of collagen fiber in the myocardium at 8 weeks after gel implantation. A and C, Representative pictures of Col1 (A) and Col3 (C) immunohistochemical staining in the infarcted area. B and D, Percentage of sections immunopositive for Col1 (B) and Col3 (D) in the infarcted area. E and G, Representative pictures of Col1 (E) and Col3 (G) immunohistochemical staining in the infarcted area. F and H, Percentage of sections immunopositive for Col1 (F) and Col3 (H) in the infarcted border area. I and J, Ratio of Col1 to Col3 in the infarcted area (I) and the infarcted border area (J). $n = 5$ in each group. $**P < .01$ versus control. *Col1*, Collagen type I; *N-OPN*, N-terminal osteopontin; *SV*, SVVYGLR sequence; *RGD*, Arg-Gly-Asp; *Col3*, collagen type III.

cardiac function. The differences in %FS and EF among the groups over time are not very large; however, they indicate a significant improvement in cardiac function owing to Col3, and show the therapeutic effects of increasing the ratio of Col3 to Col1.

We also produced recombinant proteins corresponding to the thrombin-cleaved human N-OPN as well as N-OPN without RGD and/or SVVYGLR motifs, which were used to investigate their activity on key players in cardiac function and LV remodeling, with an emphasis on collagen distribution. Numerous studies have analyzed the distribution of OPN in various tissues, showing that it functions either as a proinflammatory cytokine or as ECM.¹²⁻¹⁶ OPN contains an RGD site that mediates its attachment to integrin and a CD44-binding domain.^{12,28,29} Furthermore, OPN has a thrombin cleavage site at ¹⁶⁸RS¹⁶⁹ that is immediately downstream of the ¹⁵⁹RGD¹⁶¹.^{16,17} Thrombin cleavage exposes a C-terminal ¹⁶²SVVYGLR¹⁶⁸ site in the cleaved OPN that interacts with $\alpha_9\beta_1$ ^{16,17,30} as well as transforming growth factor (TGF)- β receptor.¹⁹ Our previous study revealed that thrombin-cleaved N-OPN and SV peptides promoted the migration of cardiac fibroblasts to the fibrotic area, where the migrated cardiac fibroblasts differentiated into myofibroblasts and produced collagen, especially Col3, via TGF- β /Smad signaling in vitro and maintained cardiac function in a rat ICM model for 4 weeks.¹⁸ However, our previous studies did not include a detailed evaluation of the N-OPN motif(s) important for these effects or the impact of increased Col3 expression on cardiac function mediated by this fragment in vivo.

The RGD sequence is present in many ECM proteins and can interact with integrin at focal adhesion points.^{12,31} This interaction activates signal transduction between the cell and the ECM, thereby influencing cell motility, including proliferation, differentiation, migration, and survival. However, in the present study, the fragment containing the SVVYGLR motif and SV peptide significantly inhibited cardiac depression and increased Col3 expression in impaired myocardium, whereas the Δ SV fragment had no effect on these changes. This implies that the SVVYGLR motif, rather than the RGD motif, played a key function in the effects of N-OPN on cardiac function and LV remodeling. Furthermore, the N-OPN fragments containing the SVVYGLR motif and SV peptide caused the increase of Col3 expression and altered the ratio of Col1 to Col3. These results also demonstrate that this change in collagen distribution and the reduced ratio of Col1 to Col3 likely have crucial roles in the attenuation of cardiac dysfunction mediated by these fragments.

Both Col1 and Col3 have been shown to be closely associated with myocardial fibrosis, and their levels are increased in the LV remodeling process.³² The contractility of the myocardium is determined by the viscoelasticity and relative content of Col1 and Col3.³² The degree of cross-

linking and the ratio of newly synthesized Col1 and Col3 have important roles in cardiac function. Thus, our study evaluated the ratios among collagen types, and found that cardiac function after cell sheet or gel implantation was maintained in rats with ICM.

TGF- β 1/Smad signaling induces the expression of the α -SMA gene, a well-known marker of myofibroblast differentiation.³³ The SVVYGLR motif and SV peptide can induce the differentiation of cardiac fibroblasts to myofibroblasts through binding of the TGF- β receptor.^{18,19} According to our previous studies, as the fragment containing the SVVYGLR motif and SV peptide increased SMA expression in the present study, this suggests that the SVVYGLR/Smad signal transduction via the TGF- β receptor induced a beneficial effect on cardiac function by increasing SMA-positive myofibroblast-like cells. Myofibroblasts constitute a major source of ECM proteins, especially collagen.³³ The accumulation of myofibroblasts and the changes in the amount and/or distribution of collagen by myofibroblasts accordingly affect heart function.^{33,34} Consistent with previous reports, in the present study, the accumulated myofibroblasts stimulated by SVVYGLR motif and SV peptide led to changes in collagen distribution. Furthermore, the N-OPN fragments lacking the SVVYGLR motif also caused increased expression of SMA, although the expression level was lower than in the N-OPN, Δ RGD, and random groups. Therefore, a functional site other than the SVVYGLR and RGD motifs is likely involved in the induction of SMA expression.

This study had some limitations. We found it difficult to express full-length Col3a1 by gene transfer to primary Fbs, and instead used a fragmented Col3a1, which has the same structural quality as the full-length protein. As reported previously, this fragmented Col3a1 contains the repeated Gly-X-Y structural motif that is necessary for perfect triple-helix formation, and is equivalent to full-length Col3 in terms of hydroxylation efficiency, proline and lysine residue distribution, and triple-helix conformation thermal stability.^{35,36} In addition, owing to the relatively small sample size, the individual variation within the same group was considerable. Statistical analysis showed a significant difference between Col3 or SVVYGLR motif treatment and control groups; however, the power analysis results suggest that the sample size was too small to provide sufficient statistical power to detect a significant difference, and that a relatively large sample size is needed to claim a statistical difference. Data from a larger sample could reflect the effects of Col3 and the SVVYGLR motif on cardiac remodeling and function.

CONCLUSIONS

In conclusion, in our series, the increased distribution of Col3 in injured myocardium prevented cardiac dysfunction

by changing the balance of collagen distribution and ratio in LV remodeling. The SVVYGLR motif in the thrombin-cleaved OPN has a central role in the maintenance of cardiac function, and Col3 expression plays an important role in the beneficial effects on cardiac function mediated by the OPN fragment containing SVVYGLR and SV peptides in ICM. Some previous studies have reported the biomechanical effects of Col3 in pulmonary abnormalities and urinary bladder filling.^{37,38} Based on the effects of Col3 in this study, we consider that the elasticity of Col3 can be applied to therapeutic cardiac biomechanics.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: collagen type III, remodeling, ischemic cardiomyopathy

APPENDIX. SUPPLEMENTAL METHODS

Isolation and Culture of Primary Cardiac Fibroblasts

Primary adult rat ventricular fibroblasts (Fbs) were incubated with 0.2% collagenase type II (Worthington Biochemical, Lakewood, NJ) and 0.1% trypsin at 37°C for 10 minutes under constant shaking. Isolated cells were pelleted at the end of each 10-minute digestion period by centrifugation. Cells from the second to ninth digestions were plated on 100-mm culture dishes in Dulbecco's modified Eagle's medium (Nihonseiyaku, Tokyo, Japan) containing 10% fetal bovine serum. The isolated cells were immunofluorescently stained with anti-fibronectin antibody (DAKO, Carpinteria, Calif) to confirm the density or uniqueness of fibroblasts from the suspended cell pellet. The density of fibroblast was roughly 80%-90%.

Cell Sheet Preparation

The primary Fbs at a density of 3×10^6 cells/dish were seeded in a temperature-responsive culture dish (UpCell; CellSeed, Tokyo, Japan) and incubated until a confluent culture was obtained at 37°C. The cell sheet was harvested by lowering the culture temperature to 20°C.

Transplantation of Fb Sheets and Collagen Gel

We opened the cardiac membrane and placed the sheets over the scared area without the use of suture. With a temperature-responsive cell culture dish, cell sheets consisting of several million cells can be readily manufactured with no added scaffold material. These cell sheets maintain cell-cell and cell-ECM adhesion, because the proteases like trypsin are not used when they are collected. Thus, the implanted sheets adhere to myocardium immediately and do not fall off after chest closure. Three fibroblast sheets

were applied sequentially to the surface of the infarcted myocardium. Once adherence of the first sheet was confirmed, subsequent sheets were applied using the same technique. The implanted sheets were stacked on the infarcted myocardium.

The recombinant OPN or SV peptide-impregnated gels were transplanted onto the LV anterior wall of a rat with MI, covering the whole infarcted anterior wall. Implanted gels were approximately 1 cm². The pericardium was overwrapped by suturing with 8-0 Prolene polypropylene suture (Ethicon, Somerville, NJ) to fix to the epicardial surface. Because the infarcted area showed macroscopically whitish myocardium, we implanted sheets or gel onto the area to not contact with the border zone or remote LV tissue.

Histological Analysis

The extent of LV dilation was calculated as LV chamber diameter/interventricular septal thickness. The percentage of fibrosis was quantitatively analyzed by point counting. Cardiomyocyte hypertrophy was evaluated by measuring the 2-point shortest axes at the level of the nucleus. Immunohistochemical staining with antibodies against Col1 (Col1; Abcam, Cambridge, UK) and Col3 (monoclonal anti-Col3 antibody for analysis of gel implantation; Novus Biologicals, Littleton, Colo or polyclonal anti-Col3 antibody for analysis of cell sheet implantation; Proteintech Group, Rosemont, Ill) was also performed to assess of the level of collagen expression. The distribution of collagen was described as Col1- or Col3-positive area/field of view $\times 100$ (%). The sections were incubated with monoclonal mouse anti- α -SMA (DAKO) to detect myofibroblast accumulation. All image analyses were performed using Scion Image software (National Institutes of Health, Frederick, Md).


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MMSFVQKGSWLLALLHPTIILAQQEAVEGGCSHLGQSYADRDV
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EAGIPGVPAKGEDGKDGSPGEPGANGLPGAAGERGAPGFRGPAGPNIPGEKGPAGE
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AEKKHVWFGESEMDGGFQFSYGNPELPEDVLDVQLAFLRLSSRASQNITYHCKNSIAY
MDQASGNVKKALKLMGSNEGEFKAEGNSKFTYTILEDGCTKHTGEWSKTVFEYRTRKA
VRLPIVDIAPYDIGGPDQEFQVGVGPVCF

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aa155-378

aa1050-1220

FIGURE E1. DNA sequence of the synthetic human Col3 α 1 chain (COL3A1) DNA construct.

TABLE E1. Echocardiographic assessment of LVDd and LVDs over time by after sheet implantation

Parameter	Baseline	2 Wk	4 Wk
LVDd, mm			
Control	0.739 $\pm$ 0.107	0.749 $\pm$ 0.112	0.753 $\pm$ 0.118
Normal Fb	0.756 $\pm$ 0.094	0.756 $\pm$ 0.099	0.746 $\pm$ 0.125
Col3 Fb	0.724 $\pm$ 0.011	0.716 $\pm$ 0.092	28.68 $\pm$ 1.66*,†
LVDs, mm			
Control	0.593 $\pm$ 0.089	0.620 $\pm$ 0.087	0.644 $\pm$ 0.097
Normal Fb	0.620 $\pm$ 0.068	0.663 $\pm$ 0.111	0.661 $\pm$ 0.128
Col3 Fb	0.599 $\pm$ 0.092	0.593 $\pm$ 0.075	0.611 $\pm$ 0.106

Baseline is the time of transplantation, which was 2 weeks after ligation of LAD. Other times are weeks post-transplantation. LVDd, Left ventricular end-diastolic dimension; Fb, fibroblast; Col3, collagen type III; LVDs, left ventricular end-systolic dimension. * $P < .05$ versus control. † $P < .05$ versus normal Fb.

TABLE E2. Echocardiographic assessment of LVDd and LVDs over time after gel implantation

Parameter	Baseline	2 Wk	4 Wk	6 Wk	8 Wk
LVDd, mm					
Control	0.860 ± 0.149	0.895 ± 0.184	0.876 ± 0.113	0.978 ± 0.142	0.969 ± 0.228
N-OPN	0.789 ± 0.052	0.917 ± 0.131	0.957 ± 0.161	0.896 ± 0.101	0.964 ± 0.142
ΔRGD	0.822 ± 0.092	0.888 ± 0.128	0.889 ± 0.122	0.939 ± 0.104	0.943 ± 0.151
ΔSV	0.791 ± 0.081	0.802 ± 0.157	0.901 ± 0.148	0.898 ± 0.104	0.831 ± 0.178
ΔRGD-SV	0.889 ± 0.100	0.898 ± 0.095	0.932 ± 0.091	0.944 ± 0.103	0.949 ± 0.161
SV	0.858 ± 0.185	0.905 ± 0.099	0.949 ± 0.106	0.983 ± 0.170	0.965 ± 0.166
Random	0.828 ± 0.099	0.926 ± 0.096	0.908 ± 0.141	0.937 ± 0.115	0.923 ± 0.157
LVDs, mm					
Control	0.717 ± 0.126	0.753 ± 0.158	0.773 ± 0.119	0.845 ± 0.119	0.840 ± 0.196
N-OPN	0.658 ± 0.050	0.778 ± 0.108	0.799 ± 0.142	0.748 ± 0.084	0.815 ± 0.127
ΔRGD	0.700 ± 0.048	0.749 ± 0.107	0.756 ± 0.105	0.820 ± 0.110	0.805 ± 0.140
ΔSV	0.658 ± 0.068	0.668 ± 0.133	0.737 ± 0.132	0.751 ± 0.123	0.699 ± 0.154
ΔRGD-SV	0.738 ± 0.082	0.758 ± 0.081	0.794 ± 0.078	0.809 ± 0.086	0.828 ± 0.161
SV	0.713 ± 0.159	0.756 ± 0.106	0.814 ± 0.118	0.824 ± 0.143	0.814 ± 0.143
Random	0.694 ± 0.084	0.776 ± 0.087	0.779 ± 0.118	0.803 ± 0.108	0.783 ± 0.143

Baseline is time of transplantation, which was 2 weeks after ligation of LAD. Other times in weeks are post-transplantation. *LVDd*, Left ventricular end-diastolic dimension; *N-OPN*, N-terminal osteopontin; *RGD*, Arg-Gly-Asp; *SV*, SVVYGLR sequence; *LVDs*, left ventricular end-systolic dimension.