TABLE 1. Forward and reverse primers and probe

	F-primer	R-primer	Probe
Human			
GAPDH	GAA GGT GAA GGT CGG AGT C	GAA GAT GGT GAT GGG ATT TC	CAA GCT TCC CGT TCT CAG CC
HGF	ATG ATG TCC ACG GAA GAG GAG A	CAC TCG TAA TAG GCC ATC ATA GTT GA	TGC AAA CAG GTT CTC AAT GTT TCC CAG C
VEGF	GAA GTG GTG AAG TTC ATG GAT GTC T	CAC ACA GGA TGG CTT GAA GAT G	TTC CAG GAG TAC CCT GAT GAG ATC GA
SDF-1	CAT GCC GAT TCT TCG AAA GC	CTA CAA TCT GAA GGG CAC AGT TTG	TGT TGC CAG AGC CAA CGT CAA GCA
G-CSF	GCT GTG GCA CAG TGC ACT CT	CCC TGG ATC TTC CTC ACT TGC TC	CCT GCC CCA GAG CTT CCT GCT CA
Hamster			
GAPDH	CTG CAC CAC CAC CTG CTT AGC	GCC ATG CCA GTG AGC TTC C	CTG CAC CAC CAC CTG CTT AGC
HGF	AGG TCC CAT GGA TCA CAC AGA	GCC CTT GTC GGG ATA TCT TTC T	ACC AGC AGA CAC CAC ACC GGC A
VEGF	GCA CTG GAC CCT GGC TTT ACT	TCA TGG GAC TTC TGC TCT CCT T	ACC ATG CCA AGT GGT CCC AGG CT
α-Sarcoglycan	AAC TGA AGA GAG ACA TGG CCA CC	CAG TGC TGG TCC AGG ATG AGG	CCT CTC TCC ACC TTG CCC ATG TTC A
β-Sarcoglycan	TCC ACT GAG AGG ATT ACC AGC AAT	AGT TTG TAG CGC ACC CAG TCA C	TCC TCA ATG GAA CTG TGA TGG TCA GCC C
α-Dystroglycan	CAC ACA GTC ATT CCA GCT GTT GT	TCA TCC AGC TCG TCT GCA AAG	CCT TGA GGA CCA GGC CAC CTT TAT CAA

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *HGF*, hepatic growth factor; *VEGF*, vascular endothelial growth factor; *SDF-1*, stromal cell–derived factor-1; *G-CSF*, granulocyte colony stimulating factor.

transcriptase (Qiagen, Hilden, Germany). Quantitative polymerase chain reaction (PCR) was performed with the ABI 7500 Fast Real-Time PCR using TaqMan Universal Master Mix (Applied Biosystems, Division of Life Technologies Corporation, Carlsbad, Calif) and the designed primers/probes (Table 1). Expression of each mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Western Blotting

The LV free wall was homogenized and centrifuged at 1000 g at 4°C for 10 minutes to retrieve protein. Subsequently, 10 to 20 μg of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis with 12.5% gels and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, United Kingdom) using the Mini Trans-Blot system (170-3930; Bio-Rad Laboratories, Hercules, Calif). Protein blots in polyvinylidene difluoride membranes were incubated with each of the primary antibodies against α-sarcoglycan or GAPDH (Abcam, Plc, Cambridge, United Kingdom). The membranes were incubated with the corresponding horseradish peroxidase–labeled secondary antibody and then visualized using the ECL system (GE Healthcare Lifesciences, Cleveland, Ohio). The band intensities were quantified by Image-J software (Wine Rasband, Bethesda, Md). The values obtained were expressed as a percentage of the value in the J2N-n hamsters.

Statistical Analysis

Data are summarized as means ± SEMs. Comparisons among groups were made with the use of 1-way analysis of variance, followed by the Tukey honestly significant difference test. Differences across the whole time course of echocardiographic data and the concentration of ONO1301 were analyzed by repeated analysis of variance including main effects of the group, time, and their interactive effects, followed by pairwise comparisons at different times using a paired *t* test with the Bonferroni multiplicity correction. Survival curves were prepared by using the Kaplan-Meier method and compared using the log-rank test. All probability values are 2-sided. Statistical analysis was performed by using SPSS version 11.0 (SPSS, Inc, Chicago, Ill).

RESULTS

Effects of ONO1301 on the Secretion of Protective Factors in Vitro

Dose-dependent effects of ONO1301 on each component of the cells were assessed by cultivating commercially available NHDFs, HCoASMCs in vitro. Synthesis and extracellular secretion of protective factors were quantitatively assessed by real-time PCR and enzyme-linked immunosorbent assay (ELISA; Quantikine; R&D Systems, Minneapolis, Minn), respectively. ONO1301 treatment upregulated the expressions of HGF, VEGF, and stromal cell–derived factor-1 (SDF-1), but not granulocyte colony stimulating factor (G-CSF), in the NHDFs assessed by real-time PCR (Figure 1, *A*). In particular, a high concentration of ONO1301 significantly upregulated expressions of these factors. These findings were consistent with the results of ELISA for the culture supernatants (Figure 1, *B*). A high concentration of ONO1301 also significantly upregulated expressions of HGF, VEGF, and G-CSF, but not SDF-1, in the HCoASMCs, assessed by real-time PCR and ELISA (Figure 1, *C* and *D*).

Heart-Dominant Elevation of ONO1301 Concentration After ONO1301 Treatment

The difference in the ONO1301 concentration between the plasma and the LV after ONO1301 treatment was quantitatively and serially assessed by the LC/MS/MS method. The ventricular ONO1301 concentration was markedly greater than the plasma concentration at week 1 and week 2 after the treatment, although the plasma ONO1301 concentration

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was higher than the detectable limit for this period (Figure 2, *A*). Both ventricular and plasma concentrations of ONO1301 were less than the detectable limit at weeks 4 and 8.

Presence of IPR in the Vasculature of the IDCM Heart

Localization of the IPR in the heart was assessed by immunohistolabeling. IPR was present in the microvasculature component, such as vascular SMCs and endothelial cells (Figure 2, *B* and *C*), but not in the cardiac fibroblasts or cardiomyocytes (Figure 2, *D*). Expression of the IPR was not different between the J2N-k and J2N-n hamsters.

Preserved Cardiac Performance in the IDCM Hamster With ONO1301 Therapy

The functional effects of ONO1301-atelocollagen sheet implantation on the IDCM heart were serially assessed by transthoracic echocardiography. LVDd/Ds and EF at 20 weeks of age, just before the treatment, were not significantly different among the 3 groups (Figure 2, *E*). After treatment, echocardiography showed that the LVDd/Ds and EF were significantly preserved until 4 weeks in the O group, compared with the N and S groups, which showed a progressive increase of LVDd/Ds and a progressive reduction of LVEF for the subsequent 8 weeks. However, even the O group showed progressively increased LVDd/Ds and reduced LVEF in the subsequent 4 weeks.

Upregulated HGF and VEGF in the Heart After ONO1301 Treatment

Real-time PCR was used to quantitatively assess the trend in expression of angiogenic factors, such as HGF and VEGF, in the hearts of the 3 groups and the normal hamster (n = 5, each). Intramyocardial mRNA levels of HGF and VEGF in the S and the N groups were not significantly different from those in the normal hamster at 2 or 4 weeks (Figure 3, *A*). In contrast, in the O group, both HGF and VEGF were significantly upregulated at 2 weeks compared with the other groups. VEGF significantly upregulated in the O group at 4 weeks, although the HGF level in the O group was not significantly different from that in the other groups at 4 weeks.

Increased Vasculature in the Heart After ONO1301 Treatment

The trend in the distribution and the number of arterioles and capillaries in the heart after ONO1301 treatment was assessed by vWF-labeled sections of the 3 groups and normal hamsters (n = 5, each). The number of vWF-positive arterioles and capillaries was significantly less in the N and the S groups than in the normal heart at 2 and 4 weeks. In contrast, vWF-positive arterioles and capillaries were homogeneously increased in the O group at 2 and 4 weeks compared with the other groups (Figure 3, *B*; $P < .05$).

Effect of ONO1301 on Myocardial Fibrosis

The distribution and the quantity of interstitial collagen in the heart after ONO1301 treatment was assessed by picrosirius red–stained sections (n = 5, each). Interstitial collagen was significantly accumulated in the J2N-k hamsters, regardless of the treatment, compared with the normal hamsters (Figure 3, *C*). However, collagen accumulation was significantly less in the O group than in the S and the N groups at 2 and 4 weeks.

Reorganization of Cytoskeletal Proteins After ONO1301 Treatment

The trend in the expression of α-sarcoglycan in the hearts after ONO1301 treatment was comprehensively assessed by immunohistolabeling, real-time PCR, and Western blotting analysis (Figure 4, *A-C*). α-Sarcoglycan was homogeneously expressed around the cardiomyocytes of the normal hamster, but it was rarely expressed in the heart of the N or S groups on immunoconfocal microscopy. In contrast, α-sarcoglycan expression was greater in the O group than in the N and S groups at 2 weeks, but not at 4 weeks. The mRNA of α-sarcoglycan was significantly greater in the O group than in the S and the N groups at 2 weeks, but not at 4 weeks. Consistently, Western blotting analysis in the heart showed significantly upregulated α-sarcoglycan expression at 2 weeks, but not at 4 weeks in the O group, compared with the S and the N groups. In addition, the trends in expressions of other cytoskeletal proteins, such as β-sarcoglycan and α-dystroglycan, after the ONO1301 treatment were assessed by immunohistolabeling and real-time PCR (Figure 4, *D* and *E*). β-Sarcoglycan was rarely expressed in the J2N-k hamster heart regardless of treatment, whereas expression of α-dystroglycan appeared to be greater in the O group than in the S and the N groups, although there were no significant differences.

Survival Benefit of ONO1301 Treatment in IDCM

No mortality or morbidity related to surgical procedure was identified in any of the groups. Survival of J2N-k hamsters after treatment was then assessed using the Kaplan-Meier method. Hamsters of the N and the S groups showed similar progression to death, primarily owing to congestive cardiac failure over the 4 weeks after treatment. In contrast, survival of hamsters in the O group was significantly prolonged compared with that of the other groups (Figure 5).

DISCUSSION

Summary of the Findings

In the present study, ONO1301 induced secretion of multiple cardiac protective factors such as HGF, VEGF, SDF-1, and G-CSF from NHDFs and HCoASMCs in a dose-dependent manner in vitro. Epicardial implantation of an ONO1301-immersed atelocollagen sheet, which was developed as a slow-releasing drug delivery system, induced heart-dominant elevation of ONO1301 for 2 weeks in

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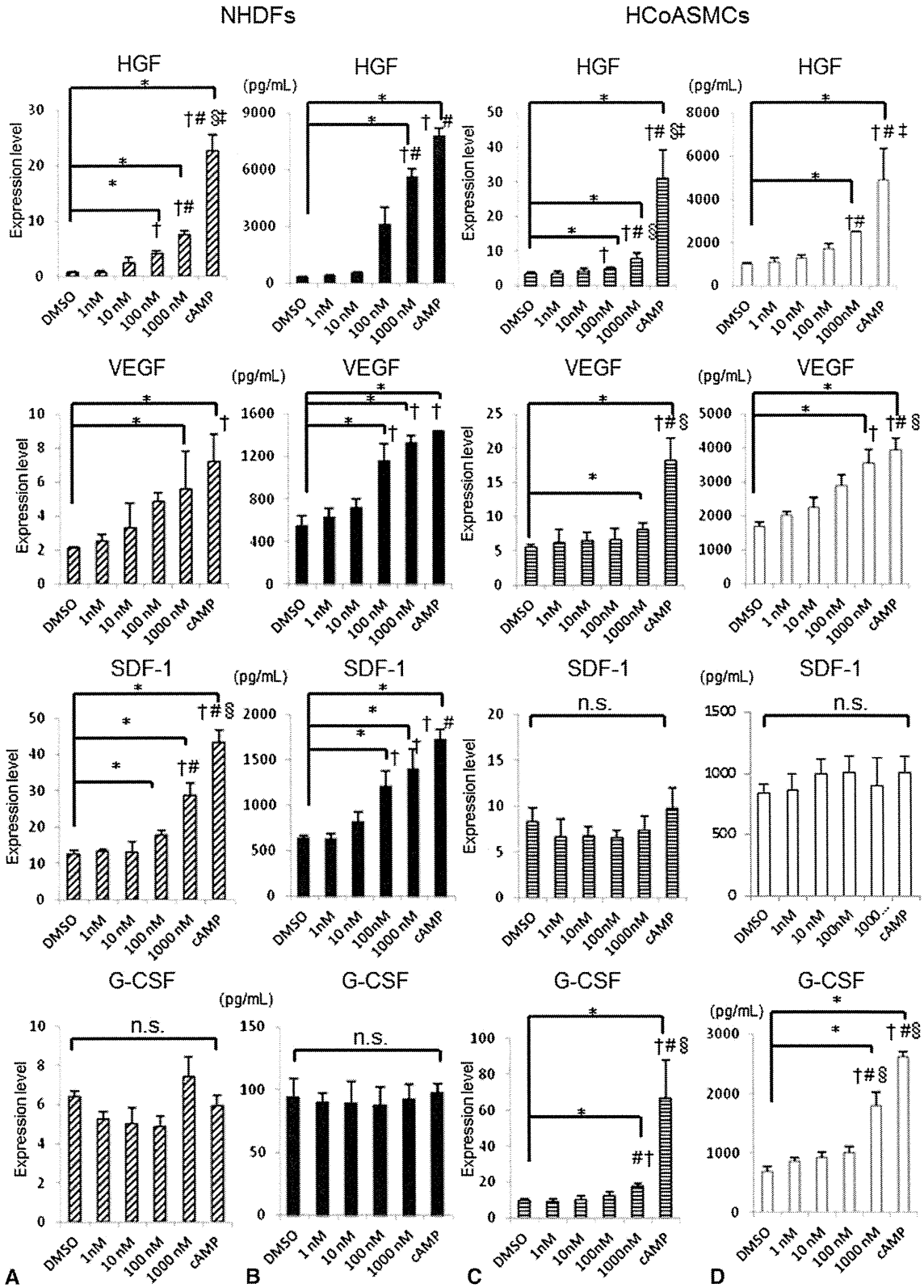

FIGURE 1. Effects of ONO1301 on the production of cardiac protective factors in NHDFs and HCoASMCs were assessed in vitro. ONO1301 treatment significantly upregulates expressions of HGF, VEGF, and SDF-1 in NHDFs, assessed by real-time PCR (A). HGF, VEGF, and SDF-1, but not G-CSF, are significantly secreted into the culture supernatant of NHDF after ONO1301 addition, measured by ELISA (B). ONO1301 addition significantly upregulates expressions of HGF, VEGF, and G-CSF, but not SDF-1, in HCoASMCs, assessed by real-time PCR and ELISA (C and D). *$P < .05$ versus control (DMSO);

the δ-sarcoglycan-deficient DCM hamsters. ONO1301-atelocollagen sheet implantation significantly upregulated expression of HGF, VEGF, increased vasculature, attenuated fibrosis, and upregulated α-sarcoglycan in the myocardium and, consequently, preserved cardiac performance and prolonged survival in this hamster DCM model.

Rationale, Feasibility, and Safety of an Atelocollagen Sheet–Based Therapy for IDCM

This study identified cell-dependent and dose-dependent effects of ONO1301 on the release of cardioprotective factors. The cells that were activated by ONO1301 in vitro included skin fibroblasts and CoASMCs. In addition, IPR, which is the sole receptor of ONO1301, was expressed in CoASMCs and endothelial cells, but not in cardiomyocytes or cardiac fibroblasts. These findings suggest that the target cells of ONO1301 may be the vascular SMCs and endothelial cells in the cardiac tissue. Local delivery of ONO1301 into the heart, directly targeting cardiac SMCs and endothelial cells, would thereby theoretically be useful in maximizing the therapeutic effects of ONO1301. In fact, it was shown that the ONO1301-immersed atelocollagen sheet implantation therapy induced marked heart-dominant elevation of the ONO1301 level in association with significantly positive functional effects, indicating rationale and feasibility of this treatment in the IDCM heart. In addition, both the ONO1301 and the atelocollagen sheet only groups did not produce procedure-related mortality despite the deteriorated cardiac function, suggesting the safety of this treatment for IDCM heart.

There are other possible clinically relevant methods for ONO1301 delivery to treat the DCM heart, such as injection of intramyocardial microbeads, systemic intravenous/subcutaneous injection, or oral intake. However, these methods are theoretically limited by possible local damage and/or poor efficiency in the drug delivery to the cardiac tissue[9] compared with the atelocollagen sheet–based drug delivery system as in this study. Intramyocardial injection of microbeads may also prove to be more efficacious, but further studies will be required to establish the optimal delivery methods of ONO1301 into the heart in preclinical and subsequent clinical studies.

Therapeutic Effects and Underlying Mechanisms of ONO1301 Sheet Therapy

Therapeutic efficiency of this treatment on this δ-sarcoglycan-deficient hamster IDCM model was assured in this study by comparing with the 2 control groups, in which the sham operation or placement of atelocollagen sheet only was performed. Global systolic cardiac function, assessed by echocardiography, was significantly preserved in the ONO1301-treated hamsters compared with the other groups, and most important, the life expectancy of the J2N-k hamsters was prolonged by this treatment. These important positive findings would be explained by multiple fundamental effects of this treatment, including increased myocardial blood flow, reduction of myocardial fibrosis, and reorganization of cytoskeletal proteins.

It has been reported that ONO1301 acts as an inducer of multiple cardioprotective factors in ischemic cardiac diseases.[5] Effects of ONO1301 on the IDCM heart, however, are poorly understood. Although the clinical manifestations of end-stage IDCM are similar to those of end-stage ischemic cardiomyopathy, typical IDCM is characterized by a decreased vascular network, increased fibrous components, and decreased expression of cytoskeletal proteins in a global and homogeneous manner.[10,11] This study also identified multiple endogenous factors upregulated by the ONO1301-atelocollagen sheet, such as HGF, a unique growth factor with antifibrosis and angiogenesis effects,[8,12] or VEGF, an important mediator of angiogenesis.[4] In addition, SDF-1 or G-CSF by the ONO1301 treatment in this study may have contributed to therapeutic stem cell homing and activation.[13,14] Further studies will be required to determine whether these agents induce regenerative responses.

The potential effects of these endogenous factors were well correlated with the pathologic changes in this study, such as increased vasculature, attenuated fibrosis, or upregulated α-sarcoglycan. Of them, increased blood flow may be one of the major mechanisms responsible for the positive therapeutic effects of the ONO1301 in this study. It has been shown that prostacyclin and prostacyclin-inducing HGF/VEGF bring a multiplier effect of vasodilation and new vessel formation responsible to regional ischemic insult.[15,16] In addition, genetic deletion of IPR had an important role on progression of cardiovascular disease.[15]

It is also interesting that cytoskeletal proteins were remodeled by the ONO1301 treatment in this study. Immunohistolabeling in this study demonstrated the transient reexpression of α-sarcoglycan and α-dystroglycan in the O group. It was speculated that α-sarcoglycan can be recycled from the plasma membrane differently from other sarcoglycans,[17] and inhibition of Smad3 associated with transforming growth factor β signal pathway suppressed by prostacyclin or HGF, brings to α-sarcoglycan gene expression.[18]

†$P < .05$ versus ONO1301 (1 nmol/L); #$P < .05$ versus ONO1301 (10 nmol/L); §$P < .05$ versus ONO1301 (100 nmol/L); ‡$P < .05$ versus ONO1301 (1000 nmol/L). *NHDF*, Normal human dermal fibroblast; *HCoASMC*, human coronary artery smooth muscle cell; *HGF*, hepatic growth factor; *VEGF*, vascular endothelial growth factor; *SDF-1*, stromal cell–derived factor-1; *PCR*, polymerase chain reaction; *G-CSF*, granulocyte colony stimulating factor; *ELISA*, enzyme-linked immunosorbent assay; *DMSO*, dimethyl sulphoxide; *cAMP*, cyclic aminophosphatase; *n.s.*, not significant.

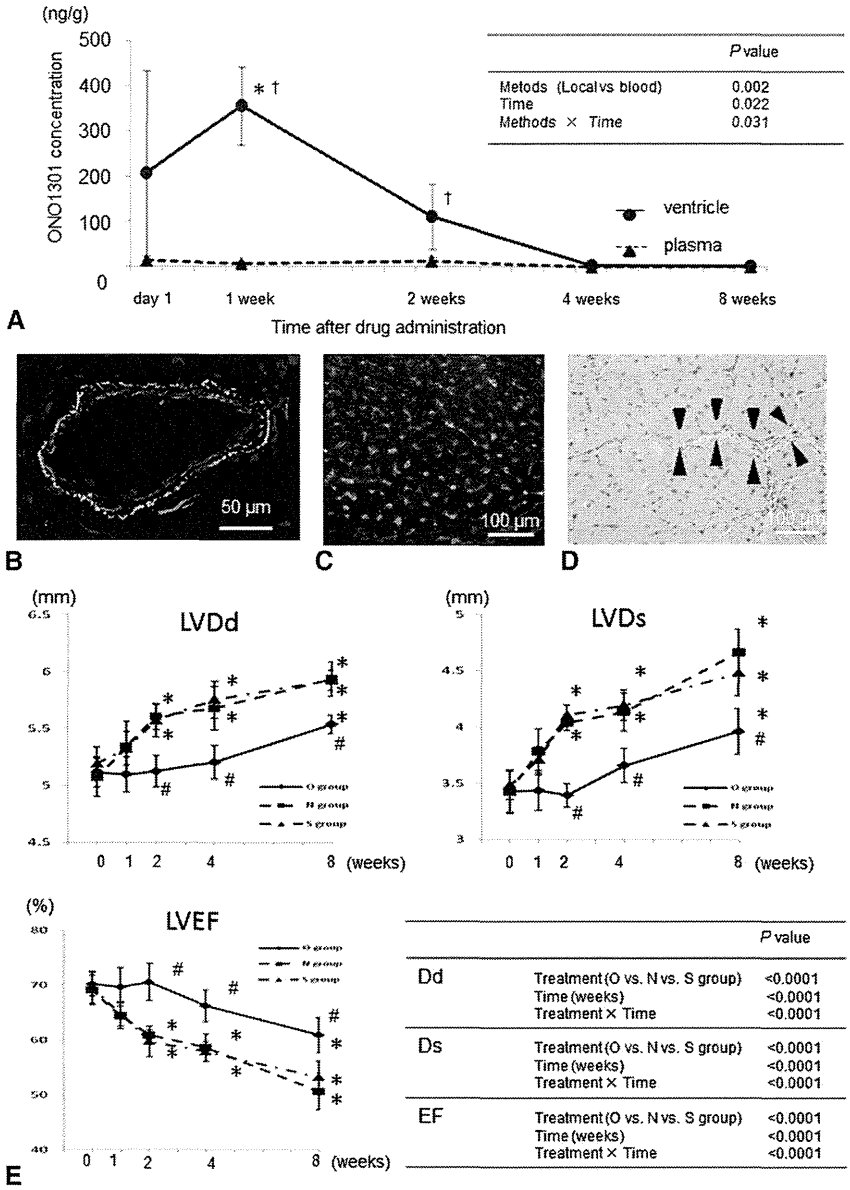

FIGURE 2. Levels of ONO1301 in cardiac tissue and plasma were serially quantified after implantation of ONO1301-eluted atelocollagen sheet for the DCM heart (A). ONO1301 is detected in both samples for 2 weeks after treatment, while the level of ONO1301 in the ventricle is significantly and markedly higher than in the plasma at weeks 1 and 2 after the treatment. *$P < .05$ versus 8 weeks; †$P < .05$ versus plasma concentration Immunofluorescence staining for IPR and alpha-actin in the DCM heart shows that IPR is positive in the vascular smooth muscle cells and the endotherial cells (B and C). *Green*, Filamentous-actin; *red*, IPR; *blue*, nuclei. 3,3′-diaminobenzidine staining, which produces a *brown color*, shows that IPR is expressed in the microvasculature, but not in cardiac fibroblasts and cardiomyocytes (*arrowhead*, cardiac fibroblast) (D). Changes in LVDd/Ds, and LVEF after treatment were serially measured by transthoracic echocardiography (E). These 4 parameters of the LV are preserved until 4 weeks after ONO1301 treatment compared with the other groups. However, ONO1301 treatment does not arrest the progression in dilatation of the dimensions and deterioration of the EF in the subsequent 4 weeks. #$P < .05$ versus N and S group; *$P < .05$ versus 0 weeks. *DCM*, Dilated cardiomyopathy; *LV*, left ventricular (ventricle); *Dd/Ds*, diastolic/systolic dimensions; *EF*, ejection fraction; *IPR*, prostacyclin receptor.

Moreover, regarding the transient reexpression of α-dystroglycan, Kondoh and associates[9] suggested that the reconstruction of α-dystroglycan may occur because the sarcoglycan might mask the matrix metalloproteinase cleavage site on dystroglycan and/or matrix metalloproteinase activity might be inhibited by HGF. In addition, β-sarcoglycan was rarely expressed after the ONO1301 treatment in our study. Kawada and colleagues[19] reported that both β- and δ-sarcoglycan were completely missing, but α- and γ-sarcoglycan were weakly expressed in the J2N-k hamster, and transfer of the δ-sarcoglycan gene could express not only δ- but the other 3 sarcoglycans. These findings might suggest the limitation of this drug therapy for reorganization of cytoskeletal proteins, but Hack and coworkers[20] reported that the

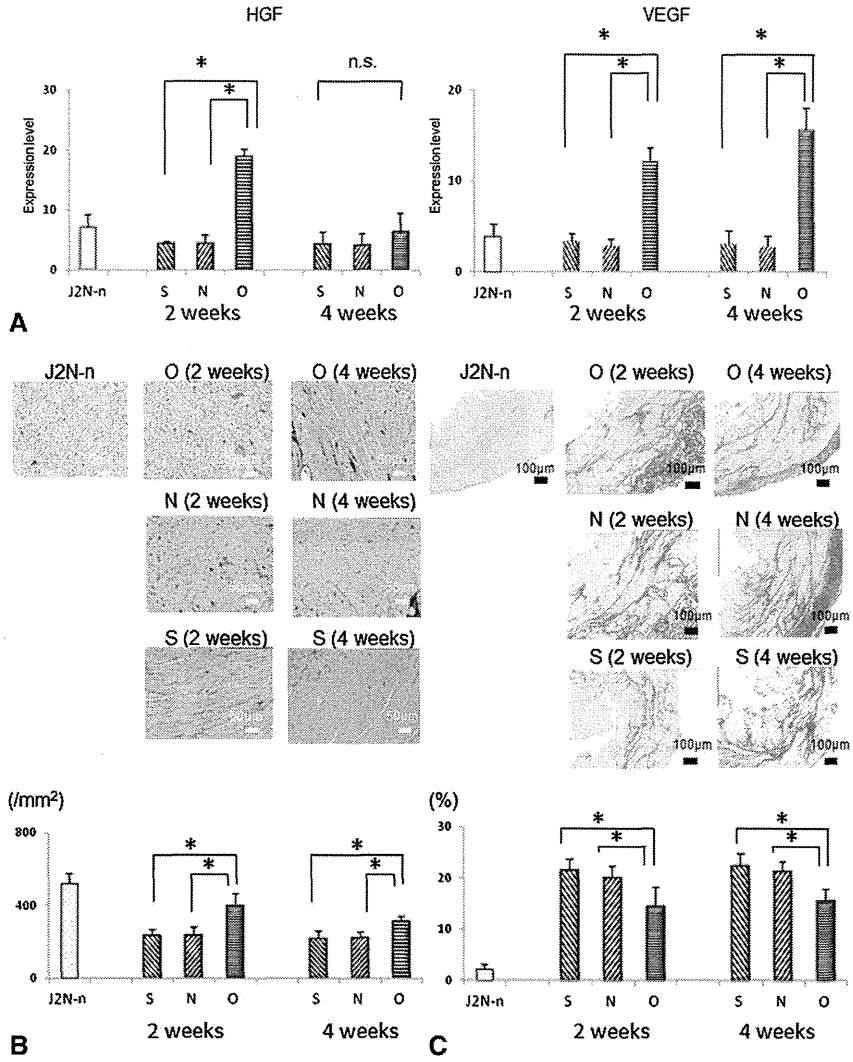

FIGURE 3. Expressions of HGF and VEGF in the cardiac tissue were assessed by real-time PCR, which shows greater expression of the 2 factors in the O group at 2 and 4 weeks compared with the other groups (n = 5 for each group) (A). Capillary density in the hearts was assessed by immunohistolabeling for vWF, which shows a greater number of capillaries in the ONO1301-treated hearts than in the other groups at 2 and 4 weeks (n = 5 for each group) (B). Interstitial fibrosis in the heart was assessed by picrosirius red staining, which shows less accumulation of fibrosis in the ONO1301-treated hearts than in the other groups at 2 and 4 weeks (n = 5 for each group) (C). *$P < .05$ versus O group. *HGF*, Hepatic growth factor; *VEGF*, vascular endothelial growth factor; *PCR*, polymerase chain reaction. *vWF*, von Willebrand factor.

expression of sarcoglycans, even in small amounts, prevented the damage of cardiomyocyte. Reorganization of α-sarcoglycan by the ONO1301 therapy might thus contribute to preserve cardiac function.

Although the level of prostacyclin in the heart in response to the ONO1301 treatment was not investigated in this study, it may be paradoxically elevated by the thromboxane synthase inhibitory activity of the ONO1301 on the heart,[21] possibly producing synergetic positive effects on the IDCM heart. In addition, it is interesting to research the involvement of the neurohormonal activations in the heart, such as adrenergic system, plasma renin activity, or endothelin, by the ONO1301 treatment.[22]

Clinical Perspectives

The atelocollagen sheet–based local ONO1301 delivery therapy globally reversed reduced vascular density, increased fibrosis, and reduced cytoskeletal proteins in the myocardium, all of which were the typical pathologic features in the human IDCM heart,[10,11] suggesting potential therapeutic benefits of this treatment for IDCM in the clinical scenario. In addition, safety of this treatment shown in this study warrants further preclinical study, including dose-response relation to explore minimum and maximal effective dose of the ONO1301 in the "GLP" standard. A very narrow dose necessary to achieve a positive response may prohibit this agent from clinical trials.

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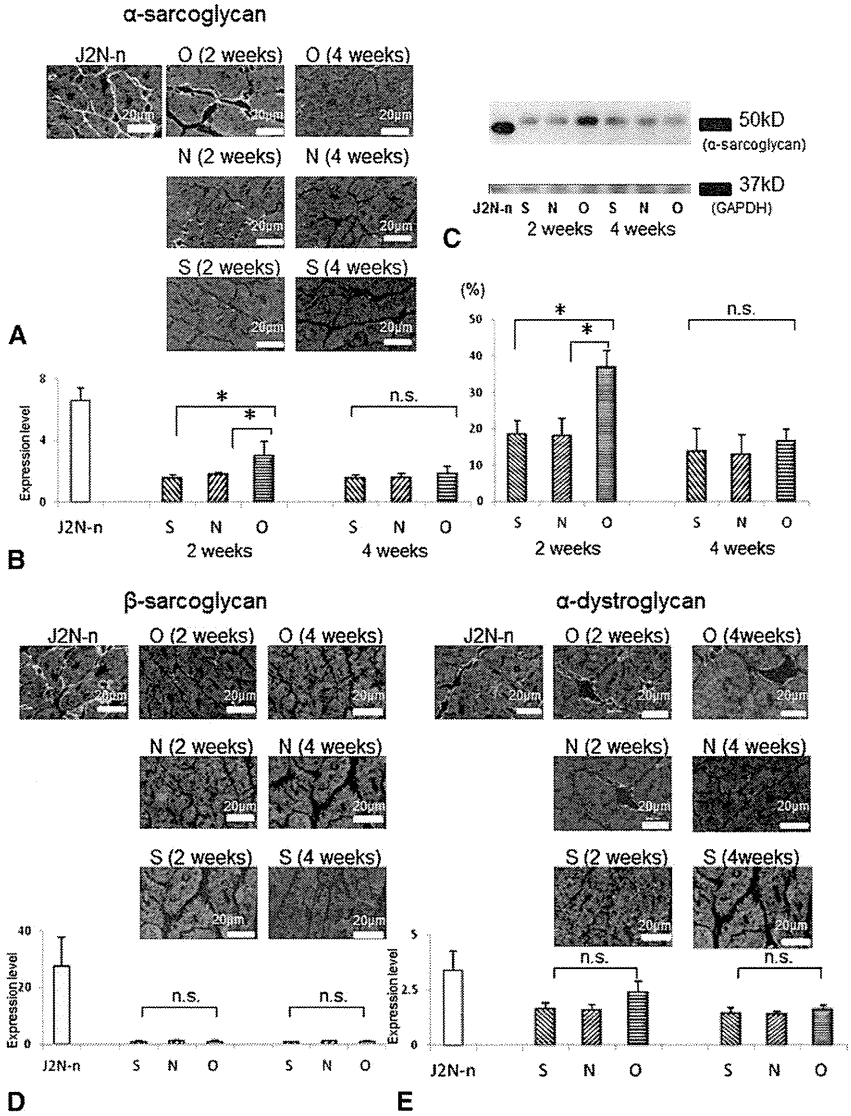

FIGURE 4. Expression of α-sarcoglycan, β-sarcoglycan, and α-dystoglycan in the heart after ONO1301 treatment was assessed by immunohistolabeling, real-time PCR, and Western blot analysis. Immunohistolabeling shows that α-sarcoglycan is clearly expressed around the cardiomyocytes in the normal hamsters, but not in the N or S group (A). Of note, α-sarcoglycan is expressed in the O group at 2 weeks but not at 4 weeks. *Green*, α-Sarcoglycan; *red*, filamentous-actin; *blue*, nuclei. Quantitative real-time PCR shows a significantly greater expression of α-sarcoglycan in the O group than in the N or S group at 2 weeks, but not at 4 weeks (n = 5 for each group at each time point, $*P < .05$ vs O group) (B). Consistently, Western blot analysis and the quantitative results of band intensities, which are expressed as a percentage of the value of the J2N-n hamsters, show significantly greater expression of α-sarcoglycan in the O group than in the N or S group at 2 weeks, but not at 4 weeks (n = 5 for each group at each time point, $*P < .05$ vs O group) (C). Expression of β-sarcoglycan in the DCM hamsters is not detected even after ONO1301 treatment (D). *Green*, β-sarcoglycan; *red*, filamentous-actin; *blue*, nuclei. α-Dystroglycan is rarely expressed in the N or S group, although its expression is upregulated in the O group (E). There are no significant differences between the O group and the other groups at 2 weeks. *Green*, α-Dystroglycan; *red*, filamentous-actin; *blue*, nuclei. *PCR*, Polymerase chain reaction; *n.s.*, not significant.

Re-treatment of epicardial implantation of the sheet containing ONO1301 might be technically challenging; however, technical modulation of microsphere generation, such as gelatin hydrogel, might induce further developments to generate a longer-release drug-delivery system than the method used in the present study.[23]

Study Limitations

This study was limited by use of a transgenic rodent model. The δ-sarcoglycan-deficient IDCM model used in this study is not completely relevant to human IDCM that shows a number of etiologic and pathologic variations. However, positive functional and pathologic effects

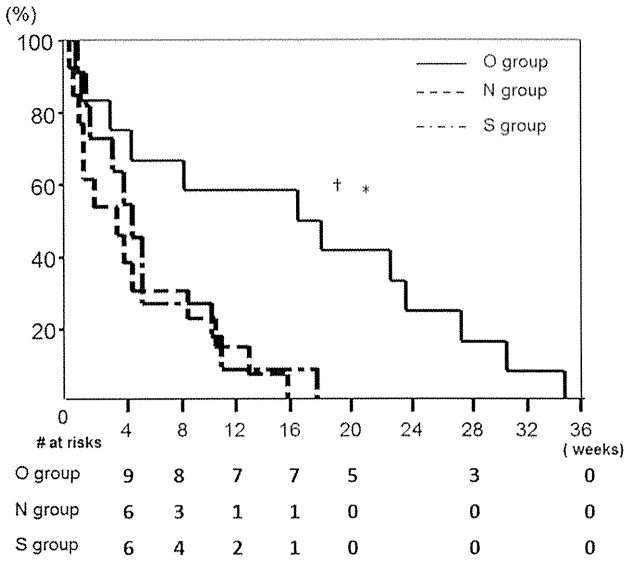

FIGURE 5. Survival after treatment was assessed by the Kaplan-Meier method. There is no significant difference between the N (n = 11) and S groups (n = 13), whereas the O group (n = 12) shows a significantly greater survival than the other groups (*$P < .05$ vs S group; †$P < .05$ vs N group).

associated with upregulated protective factors would be sufficient to prove the principal concept of this treatment. Agents that are beneficial in these mutant hamsters may not be beneficial in humans because the mechanisms responsible for the beneficial effects may be different in humans. However, further pathologic and functional studies for human DCM heart samples, in comparison with the deficient hamster, may be useful to strengthen the findings of this study.

Poor availability of the antibodies and genome sequences in the hamster limited in-depth evaluation of the mechanisms responsible for this treatment, which is warranted to be supplemented by murine IDCM model.[24]

CONCLUSIONS

ONO1301 reorganized cytoskeletal proteins, especially α-sarcoglycan, increased capillaries, and reduced fibrosis through the upregulation of cardiac protective factors, leading to functional recovery and prolonged survival in the δ-sarcoglycan-deficient IDCM hamster. A preclinical study to explore the optimal, clinically relevant protocol is warranted.

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References

1. Rajnoch C, Chachques JC, Berrebi A, Bruneval P, Benoit MO, Carpentier A. Cellular therapy reverses myocardial dysfunction. *J Thorac Cardiovasc Surg.* 2001; 121:871-8.
2. Nakamura T, Matsumoto K, Mizuno S, Sawa Y, Matsuda H, Nakamura T. Hepatocyte growth factor prevents tissue fibrosis, remodeling, and dysfunction in cardiomyopathic hamster hearts. *Am J Physiol Heart Circ Physiol.* 2005;288:H2131-9.
3. Juan CC. Cellular cardiac regenerative therapy in which patients? *Expert Rev Cardiovasc Ther.* 2009;7:911-9.
4. Matsumoto K, Okazaki H, Nakamura T. Novel function of prostaglandins as inducers of gene expression of HGF and putative mediator of tissue regeneration. *J Biochem.* 1995;117:458-64.
5. Iwata H, Nakamura K, Sumi M, Ninomiya M, Sakai Y, Sata M, et al. Local delivery of synthetic prostacycline agonist augments collateral growth and improves cardiac function in a swine chronic cardiac ischemia model. *Life Sci.* 2009;85:255-61.
6. Mitsuhashi S, Saito N, Watano K, Igarashi K, Tagami S, Kikuchi K, et al. Defect of Delta-sarcoglycan gene is responsible for development of dilated cardiomyopathy of a novel hamster strain, J2N-k: calcineurin/PP2B activity in the heart of J2N-k hamster. *J Biochem.* 2003;134:269-76.
7. Saini SG, Wani AT, Vashney B, Ahmed T, Rajan SK, Paliwal LJ. Validation of the LC-MS/MS method for the quantification of mevalonic acid in human plasma and determination of the matrix effect. *J Lipid Res.* 2006;47:2340-5.
8. Miyagawa S, Sawa Y, Taketani S, Kawaguchi N, Nakamura T, Matsuda H, et al. Myocardial regeneration therapy for heart failure: hepatocyte growth factor enhances the effect of cellular cardiomyoplasty. *Circulation.* 2002; 105:2556-61.
9. Kondoh H, Sawa Y, Miyagawa S, Matsumiya S, Sakakida-Kitagawa S, Matsuda H, et al. Longer preservation of cardiac performance by sheet-shaped myoblast implantation in dilated cardiomyopathic hamsters. *Cardiovasc Res.* 2006;69:466-75.
10. Neglia D, Michelassi C, Trivieri MG, Sambuceti G, Giorgetti A, Parodi O, et al. Prognostic role of myocardial blood flow impairment in idiopathic left ventricular dysfunction. *Circulation.* 2002;105:186-93.
11. Towbin JA. The role of cytoskeletal proteins in cardiomyopathies. *Curr Opin Cell Biol.* 1998;10:131-9.
12. Taniyama Y, Morishita R, Aoki M, Hirooka K, Yamasaki K, Ogihara T, et al. Angiogenesis and antifibrotic action by hepatocyte growth factor in cardiomyopathy. *Hypertension.* 2002;40:47-53.
13. Saxena A, Fish JE, White MD, Yu S, Smyth JW, Srivastava D, et al. Stromal cell–derived factor-1α is cardioprotective after myocardial infarction. *Circulation.* 2008;117:2224-31.
14. Stanford SJ, Pepper JR, Mitchell JA. Release of GM-CSF and G-CSF by human arterial and venous smooth muscle cell: differential regulation by COX-2. *Br J Pharmacol.* 2000;129:835-8.
15. Fetalvero KM, Martin KA, Hwa J. Cardioprotective prostacyclin signaling in vascular smooth muscle cell. *Prostaglandins Other Lipid Mediat.* 2007;82: 109-18.
16. Hiraoka K, Koike H, Yamamoto S, Tomita N, Yokoyama C, Morishita R, et al. Enhanced therapeutic angiogenesis by cotransfection of prostacyclin synthase gene or optimization of intramuscular injection of naked plasmid DNA. *Circulation.* 2003;108:2689-96.
17. Draviam RA, Wang B, Shand SH, Xiao X, Watkins SC. Alpha-sarcoglycan is recycled from the plasma membrane in the absence of sarcoglycan complex assembly. *Traffic.* 2006;7:793-810.
18. Hernández-Hernández JM, Delgado-Olguín P, Aguillón-Huerta V, Furlan-Magaril M, Recillas-Targa F, Coral-Vázquez RM. Sox9 represses alpha-sarcoglycan gene expression in early myogenic differentiation. *J Mol Biol.* 2009;394:1-14.
19. Kawada T, Nakaturu Y, Sakamoto A, Koizumi T, Shin WS, Toyooka T, et al. Strain- and age-dependent loss of sarcoglycan complex in cardiomyopathic hamster hearts and its re-expression by delta-sarcoglycan gene transfer in vivo. *FEBS Lett.* 1999;458:405-8.
20. Hack AA, Lam MY, Cordier L, Shoturma DI, Ly CT, Hadhazy MA. Differential requirement for individual sarcoglycans and dystrophin in the assembly and function of the dystrophin-glycoplotein complex. *J Cell Sci.* 2000; 113:2535-44.
21. Yamanaka S, Miura K, Yukimura T, Okumura M, Yamamoto K. Putative mechanism of hypotensive action of platelet-activating factor in dogs. *Circ Res.* 1992; 70:893-901.
22. Somova LI, Mufunda JJ. Renin-angiotensin-aldosterone system and thromboxane A2/prostacyclin in normotensive and thromboxane A2/prostacyclin in normotensive and hypertensive black Zimbabweans. *Ethn Dis.* 1992;2:27-34.
23. Takaoka R, Hikasa Y, Hayashi K, Tabata Y. Bone regeneration by lactoferrin released from a gelation hydrogel. *J Biomater Sci Polym Ed.* 2010;22:1581-9.
24. Lu D, Ma Y, Zhang W, Bao D, Dong W, Zhang L, et al. Knockdown of cytochrome P450 2E1 inhibits oxidative stress and apoptosis in the cTnT (R141W) dilated cardiomyopathy transgenic mice. *Hypertension.* 2012;60:81-9.

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A slow-releasing form of prostacyclin agonist (ONO1301SR) enhances endogenous secretion of multiple cardiotherapeutic cytokines and improves cardiac function in a rapid-pacing–induced model of canine heart failure

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Objectives: Cardiac functional deterioration in dilated cardiomyopathy (DCM) is known to be reversed by intramyocardial up-regulation of multiple cardioprotective factors, whereas a prostacyclin analog, ONO1301, has been shown to paracrinally activate interstitial cells to release a variety of protective factors. We here hypothesized that intramyocardial delivery of a slow-releasing form of ONO1301 (ONO1301SR) might activate regional myocardium to up-regulate cardiotherapeutic factors, leading to regional and global functional recovery in DCM.

Methods and Results: ONO1301 elevated messenger RNA and protein level of hepatocyte growth factor, vascular endothelial growth factor, and stromal-derived factor-1 of normal human dermal fibroblasts in a dose-dependent manner in vitro. Intramyocardial delivery of ONO1301SR, which is ONO1301 mixed with polylactic and glycolic acid polymer (PLGA), but not that of PLGA only, yielded significant global functional recovery in a canine rapid pacing–induced DCM model, assessed by echocardiography and cardiac catheterization (n = 5 each). Importantly, speckle-tracking echocardiography unveiled significant regional functional recovery in the ONO1301-delivered territory, consistent to significantly increased vascular density, reduced interstitial collagen accumulation, attenuated myocyte hypertrophy, and reversed mitochondrial structure in the corresponding area.

Conclusions: Intramyocardial delivery of ONO1301SR, which is a PLGA-coated slow-releasing form of ONO1301, up-regulated multiple cardiotherapeutic factors in the injected territory, leading to region-specific reverse left ventricular remodeling and consequently a global functional recovery in a rapid-pacing–induced canine DCM model, warranting a further preclinical study to optimize this novel drug-delivery system to treat DCM. (J Thorac Cardiovasc Surg 2013;146:413-21)

Dilated cardiomyopathy (DCM) is characterized by progressive and severe deterioration of cardiac function, eventually leading to advanced heart failure necessitating surgical interventions such as cardiac transplantation[1] or mechanical assist device implantation,[2] despite maximum currently available medical therapy including angiotensin-converting enzyme inhibitor[3] or beta-blocker.[4] Despite a variety of etiologies in DCM, the diseases consistently include pathologic hypertrophy of cardiomyocytes associated with mitochondrial dysfunction, increased interstitial fibrosis, and limited regional blood flow.[5-7] Pathologic left ventricular (LV) remodeling is reportedly reversed, at least in part, by cell transplantation that intramyocardially up-regulates multiple cardiotherapeutic cytokines in a constitutive manner.[8,9] However, cell therapy is limited in the clinical arena owing to availability of cell processing center or ethical issues. Therefore, synthetic reagents that yield similar cardiotherapeutic effects to cell transplantation have been sought.

Prostacyclin is an endogenous factor released by endothelial cells, activating endothelial cells, fibroblasts, or smooth muscle cells in an autocrine and paracrine manner to release multiple growth factors or cytokines, consequently producing local and systemic anti-inflammatory, antifibrotic, proangiogenic, and antithrombotic effects. However, clinical use of synthetic prostacyclin or prostacyclin analogs, such as epoprostenol and beraprost, for chronic diseases is hampered by its chemical instability[10,11] and therefore the delivery method.

ONO1301 is a synthetic prostacyclin analog having a unique structural feature to maintain chemical stability,

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Abbreviations and Acronyms

DCM	= dilated cardiomyopathy
Dd	= end-diastolic left ventricular dimension
Ds	= end-systolic left ventricular dimension
E	= early transmitral filling wave
E′	= early diastolic velocity of the mitral annulus
EF	= ejection fraction
EDWT	= end-diastolic wall thickness
ELISA	= enzyme-linked immunosorbent assay
ESWT	= end-systolic wall thickness
HGF	= hepatocyte growth factor
LV	= left ventricular (ventricle)
mRNA	= messenger RNA
ONO1301SR	= slow releasing form of ONO1301
PCR	= polymerase chain reaction
PLGA	= polylactic and glycolic acid polymer
SDF-1	= stromal-derived factor-1
VEGF	= vascular endothelial growth factor

possibly allowing slow-releasing system.[12] Of note, ONO1301 reportedly activates fibroblasts to release multiple factors such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF),[13] both of which are known to be cardiotherapeutic.[14,15] Nakamura and associates[13] reported that direct intramyocardial injection of ONO1301 yielded cardiotherapeutic effects in a model of acute myocardial infarction in the mouse. On the other hand, Hirata and colleagues[16] reported that subcutaneous injection of ONO1301 improves global cardiac function associated with globally reduced fibrosis and increased capillaries in a hamster DCM model. However, it remains unclear that intramyocardial delivery of ONO1301 would produce therapeutic effects on a model of DCM heart failure in a large animal.

We therefore hypothesized that intramyocardial injection of ONO1301 might activate regional interstitial cells including fibroblasts in the injected area to locally upregulate multiple therapeutic factors, leading to region-specific functional recovery in DCM. Thus, we investigated therapeutic effects of local administration of a slow-releasing form of ONO1301 on regional cardiac function of DCM heart by using the canine rapid-pacing induction that is an established DCM model.[17,18]

METHODS

Animal Care

All studies were performed with the approval of the institutional ethics committee in Osaka University Graduate School of Medicine. All animals were treated in compliance with the "Principles of Laboratory Animal Care" (the National Society for Medical Research) and the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication). Human dermal fibroblasts were treated in compliance with the principles outlined in the Declaration of Helsinki. All procedures and analysis were carried out in a blinded manner. We had full access to and take full responsibility for the integrity of data and agree to the manuscript as written.

Culture of Human Dermal Fibroblasts With ONO1301 Added

Human dermal fibroblast cell line (NHDF; CryoNHDF Neo, Lonza, Switzerland) was cultured in fine bubble mixing culture (FGM-2 Bulletkit, Lonza) containing 2% fetal bovine serum. ONO1301 (0.1-1.0 μmol/mL) was added for 72 hours after serum-free culture for 24 hours.

Generation of a Slow-Releasing Form of ONO1301

A slow releasing form of ONO1301 (ONO1301SR; Ono Pharmaceutical Co Ltd, Osaka, Japan) was created by polymerization of ONO1301 with polylactic and glycolic acid polymer (PLGA) as described previously.[19] In brief, ONO1301 (5 mg) was mixed with 100 mg of PLGA in 0.1% polyvinyl alcohol with an equal molar ratio of lactic acid/glycolic acid. Releasing time of ONO1301SR in vitro was between about 14 days to 25 days, as determined by measuring residual ONO1301 in the pellets by liquid chromatography.

Generation of Canine DCM Model and Intramyocardial ONO1301SR Injection

Beagles weighing 10 kg (Oriental Yeast Co Ltd, Tokyo, Japan) were endotracheally intubated and supported by mechanical ventilation under general anesthesia using intravenous sodium pentobarbitual (6 mg/kg) for induction and inhaled sevoflurane (1%-2%) for subsequent maintenance. We maintained the adequacy of anesthesia evaluated by giving the dogs electrical stimuli every 30 minutes. This evaluation was performed during each operation for each procedure. The heart was exposed via the left fifth intercostal space, and 2 bipolar pacing leads (FINELINE II EZ STEROX; Boston Scientific, Boston, Mass) were attached on the free wall of the right ventricle, connected to a pulse generator (INSIGNIA I, Boston Scientific) placed in subcutaneous pocket. The ventricle was continuously paced at 240 beats/min for 8 weeks.[18]

Four weeks after rapid pacing commenced, either ONO1301SR or PLGA polymer only was injected with a 26-gauge needle at 5 points of lateral wall of the LV at regular intervals (total 15 mg of ONO1301 or PLGA polymer was injected, ONO1301SR group and control group, n = 5 each). Rapid pacing was temporally discontinued during the injection procedure, and it was set back at 240 beats/min the day after each operation. Dogs kept on rapid pacing for 8 weeks were humanely killed under general anesthesia with an overdose of intravenous sodium pentobarbitual (18 mg/kg) to achieve complete sedation followed by administration of potassium-based solution intravenously to assure that they were completely dead. The hearts were retrieved at 4 weeks after injection of either ONO1301SR or PLGA only. We here defined lateral LV wall where ONO1301SR was directly injected as the "target site" and the septal wall as the "remote site."

Conventional and Speckle-Tracking Echocardiography and Cardiac Catheterization

Transthoracic echocardiography (Altida; Toshiba Medical Systems Corporation, Tochigi, Japan) was performed under general anesthesia by 1% sevoflurane inhalation. End-diastolic and end-systolic LV dimensions (Dd and Ds, respectively) and end-systolic and end-diastolic wall thickness (ESWT and EDWT, respectively) of the target site and remote site were measured at mid-LV short axis view by conventional echocardiography. LV ejection fraction (EF) was calculated with biplanar Simpson's rule

from the apical 4-chamber view. E/E′, an indicator of diastolic function, was calculated by measuring peak Doppler velocities of early transmitral filling wave (E) and the peak early diastolic velocity of the mitral annulus (E′).

Speckle-tracking echocardiography and an offline software (Altida Extend; Toshiba Medical Systems Corporation) were used to measure radial, circumferential, transverse, and longitudinal strains to quantitatively assess regional LV wall motion.[20] Radial and circumferential strains were measured from the mid-LV short-axis view, whereas transverse and longitudinal strains were from the apical 4-chamber view.

Cardiac catheterization was performed under general anesthesia using 1% sevoflurane inhalation. A 3F micromanometer-tipped catheter (SPR-249; Millar Instruments, Inc, Houston, Tex) was inserted through the LV apex to measure heart rate, LV maximal systolic pressure, maximal rate of the LV pressure change evaluating systolic preload-dependent LV function, and time constant of LV relaxation (τ) evaluating diastolic load-dependent function.

Real-Time Polymerase Chain Reaction

Total RNA was retrieved from NHDF by using RNeasy Mini kit (Qiagen, Venlo, The Netherlands) and treated with RNase-Free DNase Set (Qiagen). TaqMan probes were designed using Primer Express software (Applied Biosystems, Carlsbad, Calif). Real-time polymerase chain reaction (PCR) was performed using a 7500 Fast Real-Time PCR System with TaqMan Universal PCR Master Mix (Applied Biosystems). Concentration of HGF, VEGF, and stromal-derived factor-1 (SDF-1) in the culture supernatant of NHDF was measured by using an enzyme-linked immunosorbent assay (ELISA) kit (Procarta Cytokine Assay kit, Panomics, Santa Clara, Calif).

Histologic Analysis and Electron Microscopy

The extracted dog hearts were transversely cut, fixed with 10% buffered formalin, and embedded in paraffin. The heart sections of 10-μm thickness were stained with hematoxylin and eosin, Masson-trichrome, picro-sirius red, and periodic acid–Schiff. The heart sections were also labeled by anti-von Willebrand factor antibody (Dako EPOS) visualized by horseradish peroxidase (DakoCytomation, Glostrup, Denmark). Fibrotic area was calculated in the picro-sirius red–stained sections by using a planimetric method with a morphometry analyzer (NIS elements D, Nikon, Japan) on 5 optical fields that were selected randomly for each sample. Extracted dog heart tissues were fixed with 2.5% glutaraldehyde, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Statistical Analysis

All data are presented as the mean ± standard error of the mean. The analyses were performed using nonparametric methods because the sample sizes were too small to allow checking of the assumptions of parametric methods. Expression of messenger RNA (mRNA) in vitro analyzed by PCR and ELISA was analyzed by Jonckheere-Terpstra test for assuring dose-dependent effect of ONO1301. Hemodynamic data obtained from conventional echocardiography, cardiac catheterization, and speckle-tracking echocardiography, as well as histopathologic findings such as percent fibrosis, cell diameter, and vascular density at the target and remote sites of control group or ONO1301SR group, were analyzed by nonparametric repeated-measures analysis. Statistical analyses were performed with the R program (R Development Core Team 2011). R: A language and environment for statistical computing. R Foundation for Statistical Computing; Vienna, Austria).

RESULTS

Effects of ONO1301 on Expression of Endogenous Cytokines In Vitro

Effects of ONO1301 on expression of HGF, VEGF, and SDF-1 in the NHDF in vitro were examined by real-time PCR and ELISA. Relative expression of mRNA for HGF, VEGF, and SDF-1 was up-regulated in the NHDF with ONO1301 added in a dose-dependent manner (Figure 1, *A-C*), which was consistent with the release of HGF, VEGF, and SDF-1 into the supernatants (Figure 1, *D-F*).

Global Recovery of the DCM Heart by Injection of ONO1301SR

Serial changes in global systolic and diastolic cardiac function were assessed under general anesthesia by conventional echocardiography at 3 time points: 0 weeks (before commencement of rapid pacing), 4 weeks after the commencement of rapid pacing (just before injection of either ONO1301SR or PLGA only), and 4 weeks after injection of either ONO1301SR or PLGA only. Cardiac performance was markedly deteriorated, including increased Dd/Ds and E/E′ and decreased EF, ESWT, and EDWT at 4 weeks, when either ONO1301MS or PLGA only was intramyocardially injected.

At 4 weeks after PLGA injection, both systolic and diastolic cardiac functions had further deteriorated.

On the other hand, EF and ESWT/ EDWT at both the target site and remote site at 4 weeks after ONO1301SR injection were significantly greater than those at 4 weeks after PLGA injection (EF, 39% ± 1.7 % vs 19% ± 2.0%; $P < .05$; Figure 2, *E*; ESWT/EDWT at target site, $1.3 \pm 3.0 \times 10^{-2}$ vs $1.1 \pm 2.0 \times 10^{-2}$; $P = .01$; Figure 2, *C*; ESWT/EDWT at remote site, 1.2 ± 0.1 vs $1.1 \pm 3.0 \times 10^{-2}$; $P = .04$; Figure 2, *D*), although the impact of the recovery was stronger in the target site. Ds was significantly smaller after ONO1301SR injection than after PLGA injection (Ds, 23 ± 2.4 vs 31 ± 1.7 mm; $P < .05$; Figure 2, *B*), whereas Dd also showed a trend to be smaller after ONO1301SR injection than after PLGA injection (Dd, 29 ± 2.3 vs 34 ± 1.4; $P < .05$; Figure 2, *A*). E/E′ after ONO1301SR injection was significantly smaller than that after PLGA injection (E/E′, 11 ± 1.2 vs 16 ± 0.5; $P < .05$; Figure 2, *F*).

Cardiac catheterization, carried out at 4 weeks after either ONO1301SR or PLGA injection, revealed that τ was significantly smaller after ONO1301SR injection than after PLGA injection (τ, 32 ± 0.9 vs 55 ± 5.8; $P < .05$). Heart rate, LV maximal systolic pressure, and maximal rate of the LV pressure change did not show any significant difference at 4 weeks after injection of either ONO1301SR or PLGA.

Regional Functional Recovery After ONO1301SR Injection

Serial changes of regional systolic cardiac function were assessed under general anesthesia by speckle-tracking echocardiography at the same 3 time points as conventional echocardiography. At 4 weeks after the commencement of rapid pacing, all strain values at both target and remote sites were decreased compared with those

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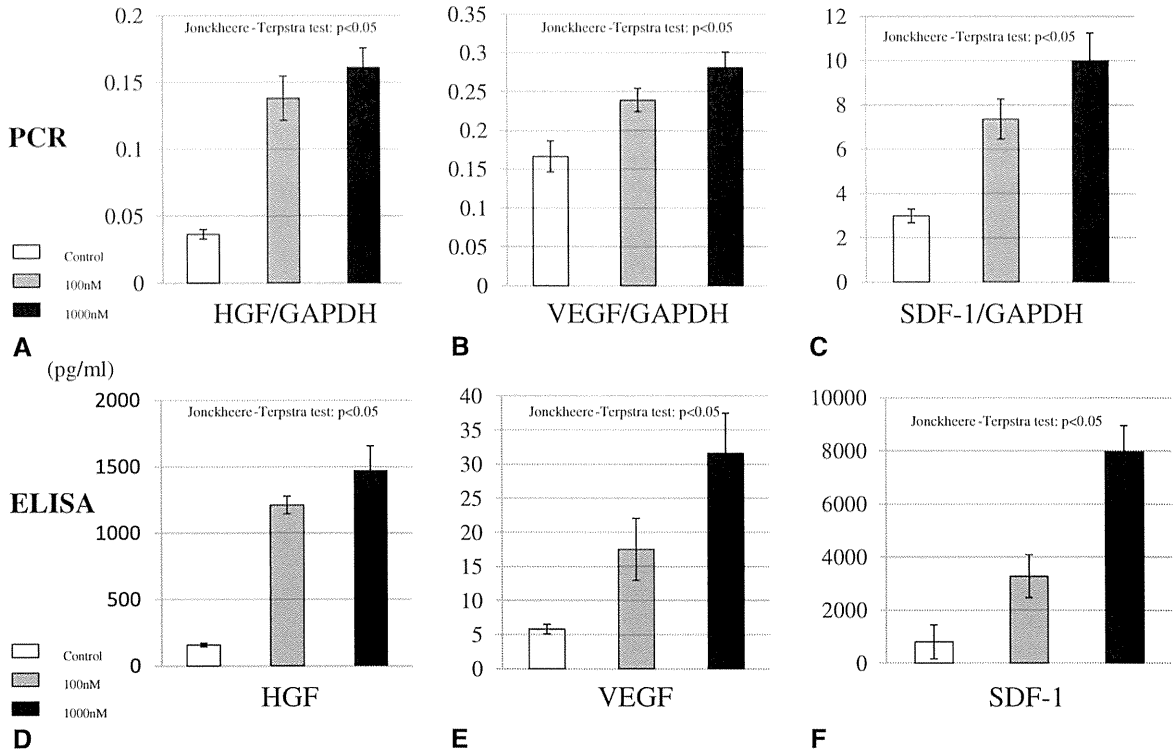

FIGURE 1. PCR and ELISA analysis in vitro showed that messenger RNA levels for HGF (A and D), VEGF (B and E), and SDF-1 (C and F) increased in a dose-dependent manner in NHDF cultured with ONO1301. *PCR*, Polymerase chain reaction; *ELISA*, enzyme-linked immunosorbent assay; *NHDF*, normal human dermal fibroblast; *HGF*, hepatocyte growth factor; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *SDF-1*, stromal-derived factor-1; *VEGF*, vascular endothelial growth factor. Mean ± standard error of the mean, respectively.

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before rapid pacing. At 4 weeks after the PLGA injection, the absolute values of peak systolic radial, circumferential, transverse, and longitudinal strains at both target and remote sites further decreased compared with those before the PLGA injection. In contrast, at 4 weeks after the ONO1301SR injection, strain values of radial, transverse, and circumferential strains were greater at the target site than those after the PLGA injection (radial strain, 36% ± 4.7% vs 8.3% ± 1.4%; $P < .05$; transverse strain, 39% ± 9.3% vs 9.5% ± 2.1%; $P < .05$; circumferential strain, −11% ± 1.3% vs −3.9% ± 0.6%; $P < .05$; Figure 3, *A-C*), although longitudinal strain was not different between the hearts with and without ONO1301SR treatment (Figure 3, *D*). On the other hand, only radial strain was significantly improved at the remote site after ONO1301SR injection compared with that after PLGA injection (Figure 3, *E-H*).

Histologic Findings of Reverse LV Remodeling After ONO1301SR Injection

Gross myocardial structure, assessed by hematoxylin and eosin staining and Masson trichrome staining, showed a thicker LV wall and a smaller LV cavity 4 weeks after ONO1301SR administration (Figure 4, *H-K*) than that after PLGA injection (Figure 4, *D-G*).

Quantity of interstitial fibrosis at the target site, evaluated by picro-sirius red staining, was significantly less at 4 weeks after ONO1301SR administration compared with that after PLGA injection (percent fibrosis at the target site, 9.9% ± 0.7% vs 23% ± 0.9%; $P < .01$; Figure 4, *A*). Of note, distribution of interstitial fibrosis was significantly more restricted at the target site than that at the remote site after ONO1301SR administration (9.9% ± 0.7% vs 16% ± 1.2%), whereas PLGA injection did not produce such an uneven distribution (23% ± 0.9% at the target site vs 23% ± 0.8% at the remote site).

Mean transverse cellular diameter of cardiomyocytes (Figure 4, *B*) at the target site, measured by periodic acid-Schiff–stained sections, was also significantly smaller at 4 weeks after ONO1301SR administration compared with that after PLGA injection (12 ± 0.6 mm vs 15 ± 0.8 mm; $P < .01$). The diameter of cardiomyocytes at the target site was smaller after ONO1301SR administration compared with that at the remote site (12 ± 0.6 vs 14 ± 0.3 mm; $P < .01$), whereas such an uneven distribution in the myocyte size was not observed after PLGA injection.

Vascular density (Figure 4, *C*), assessed by counting the number of factor VIII–positive cells in the fields, was significantly greater at the target site at 4 weeks after ONO1301SR administration compared with that after

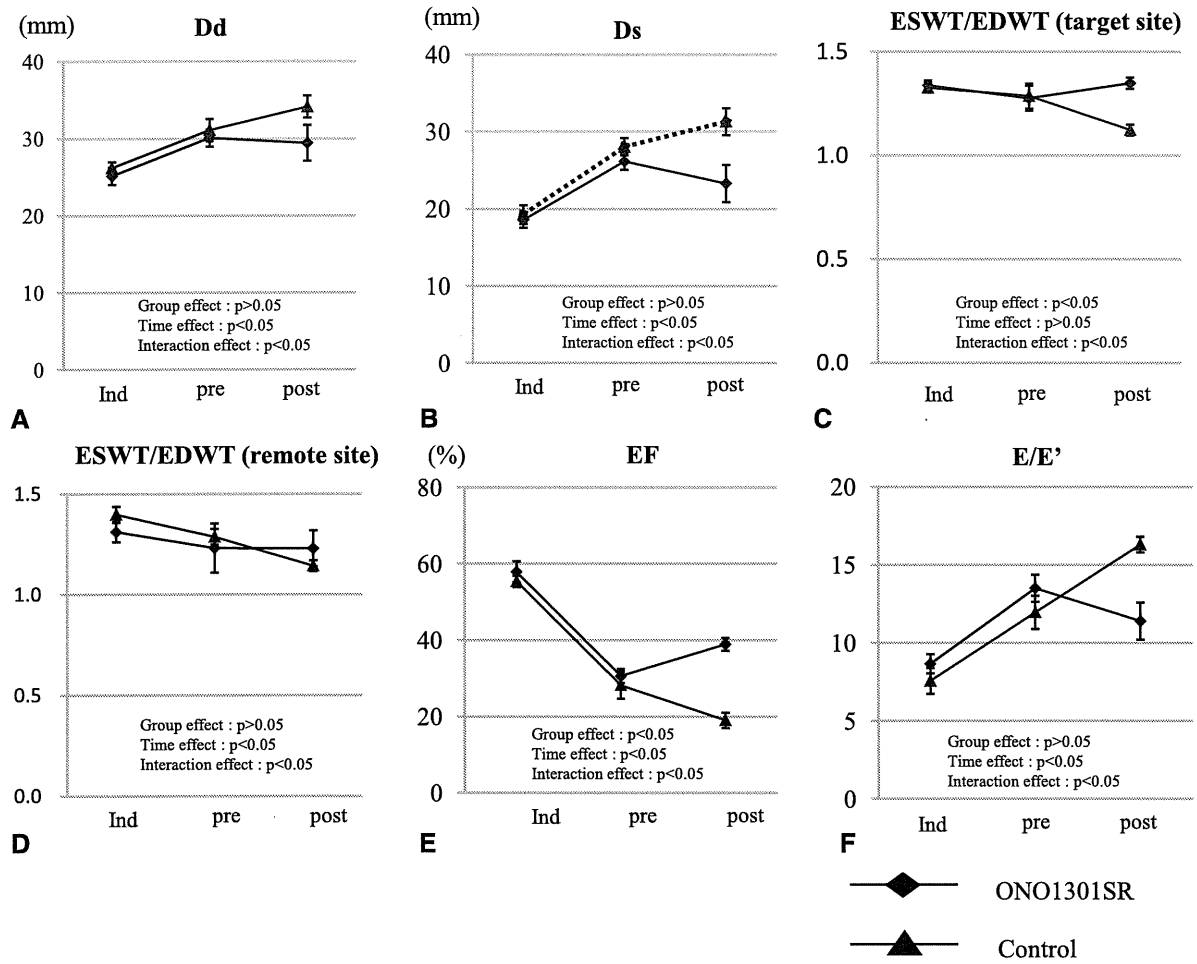

FIGURE 2. Echocardiography (A-F) showed ONO1301SR significantly improved distressed cardiac function. Note that ESWT/EDWT, reflecting on the radial strain of myocardium, was significantly more recovered in the ONO1301SR group than in the control group and that the clinical impact was more prominent in the target site after ONO1301SR treatment. *Dd*, End-diastolic dimension of the left ventricle; *Ds*, end-systolic dimension of the left ventricle; *EF*, ejection; *ESWT*, end-systolic wall thickness; *EDWT*, end-diastolic wall thickness. Mean ± standard error of the mean, respectively. *Ind*, induction of rapid pacing; *Pre*, before treatment of polylactic and glycolic acid polymer *(PLGA)* or ONO1301SR; *Post*, after treatment of PLGA or ONO1301SR.

PLGA injection (998 ± 70/mm^2 vs 467 ± 33/mm^2; $P < .01$). The vascular density at the target site was greater after ONO1301SR administration compared with that at the remote site (998 ± 70/mm^2 vs 491 ± 24/mm^2), whereas such an uneven distribution of vascular density was not observed after PLGA injection.

Electron microscopy revealed that the cardiomyocytes at 4 weeks after PLGA injection showed a prominent swelling or disruption of mitochondria, intracellular or perinuclear edema, and sarcoplasmic vacuoles resulting from dilation of sarcoplasmic reticulum (Figure 5, *A*). However, marked loss of myofilaments and alterations of characteristic sarcomeric structure were not observed in any groups. Although the interfibrillar space in the myocardium after ONO1301SR injection was slightly widened, the mitochondria were compact and showed densely packed cristae (Figure 5, *B*) compared with those after PLGA injection.

DISCUSSION

We here demonstrate that ONO1301 dose-dependently up-regulated expression of multiple cytokines, such as HGF, VEGF, and SDF-1, in fibroblasts in vitro. Histologic reverse LV remodeling, such as attenuated fibrosis and swelling of cardiomyocytes, increased vascular density, and recovered mitochondrial structure, in the target area but not significantly in the remote area, were consistent to the regional functional recovery, assessed by speckle-tracking echocardiography, which was more prominent at the target area than that at the remote area after the ONO1301SR injection. Such regional recovery at the target area after ONO1301SR injection resulted in recovery of global function, including systolic and diastolic function.

Iwata and associates[21] reported that local administration of prostacyclin analog may induce HGF production followed by VEGF expression via cyclic adenosine monophosphate–mediated pathway and that elevation of HGF

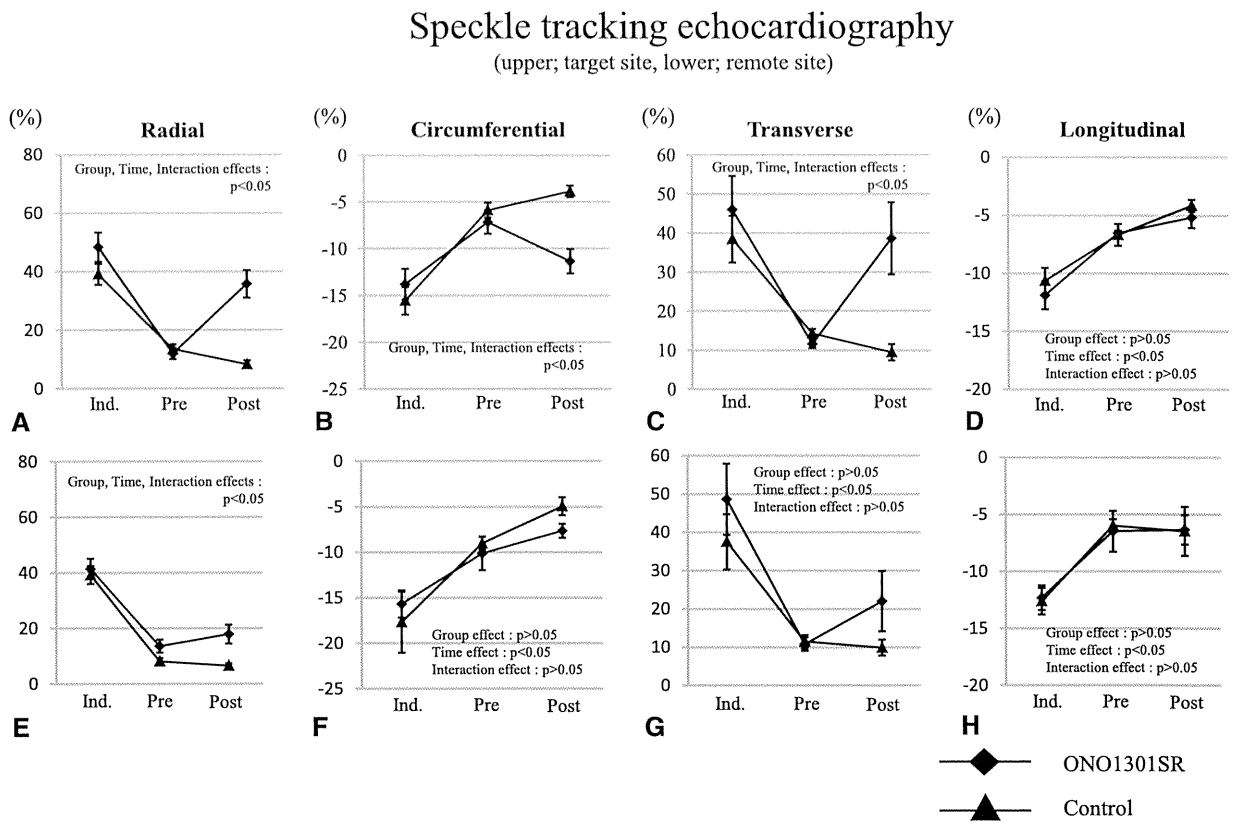

FIGURE 3. Speckle-tracking echocardiography showed that the absolute values of peak radial, circumferential, and transverse strains at the target site in the ONO1301SR group were significantly higher than in the control group (A-D) while all of them but radial strains at the remote site in the ONO1301SR group and that in the control group were not significantly different (E-H), which implied that ONO1301SR had influence on the cardiac performance especially at the very site where ONO1301SR was administered; *Radial*, radial strain; *Circumferential*, circumferential strain; *Transverse*, transverse strain; *Longitudinal*, longitudinal strain. Target site is defined as the area in which ONO1301SR or glycolic acid polymer *(PLGA)* is injected while remote site as noninjection area. *Ind*, Induction of rapid pacing; *Pre*, before treatment of PLGA or ONO1301SR; *Post*, after treatment of PLGA or ONO1301SR. Mean ± standard error of the mean, respectively.

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or VEGF may mediate the favorable effect in the treatment of ischemic heart failure. We here showed that ONO1301 directly activates fibroblasts in vitro and releases not only HGF and VEGF, as reported previously,[13,16,21] but also SDF-1, which has been thought to be a representative therapeutic stem cell homing factor in ischemic heart.[22] In the present in vivo study, we used the slow-releasing form to deliver ONO1301 and, importantly, deliver ONO1301SR directly into the myocardium of the canine DCM heart in the aim to elevate regionally ONO1301 level, thus maximizing the effects on the cardiac fibroblasts to release cardiotherapeutic factors. Consequently, pathologic and functional effects of intramyocardial ONO1301SR injection were markedly prominent in the target area (area surrounding the injection sites) compared with the remote area, suggesting that cardiac fibroblasts residing in the target area might have played a key role in locally up-regulated cardiotherapeutic cytokines.

In addition, it was noted that the typical structural features of cardiomyocytes in the severely ischemic heart, such as swelling of mitochondria, intracellular or perinuclear edema, and sarcoplasmic vacuoles referred to by a phenomenon, "permeability transition,"[23] were reversed after ONO1301SR injection in this study. On the basis of these findings, targeted injection of ONO1301SR into the damaged myocardial area might maximize therapeutic effects of ONO1301 that up-regulates cardiotherapeutic cytokines in a regional concentration-dependent manner.

Use of slow releasing form in administrating ONO1301 directly into the heart includes concerns related to the initial burst that might have an adverse effect on hemodynamics.[24] In this study, there is no hemodynamic compromise during or immediately after the procedure despite the poor cardiac function, suggesting that the protocol used here in injecting ONO1301SR might be appropriate in treating the DCM heart. Further study for dose-dependent hemodynamic change immediately after ONO1301SR administration would be needed in translating this treatment into the clinical arena.

Intramyocardial delivery of ONO1301 might be achieved by direct injection, intracoronary artery injection,

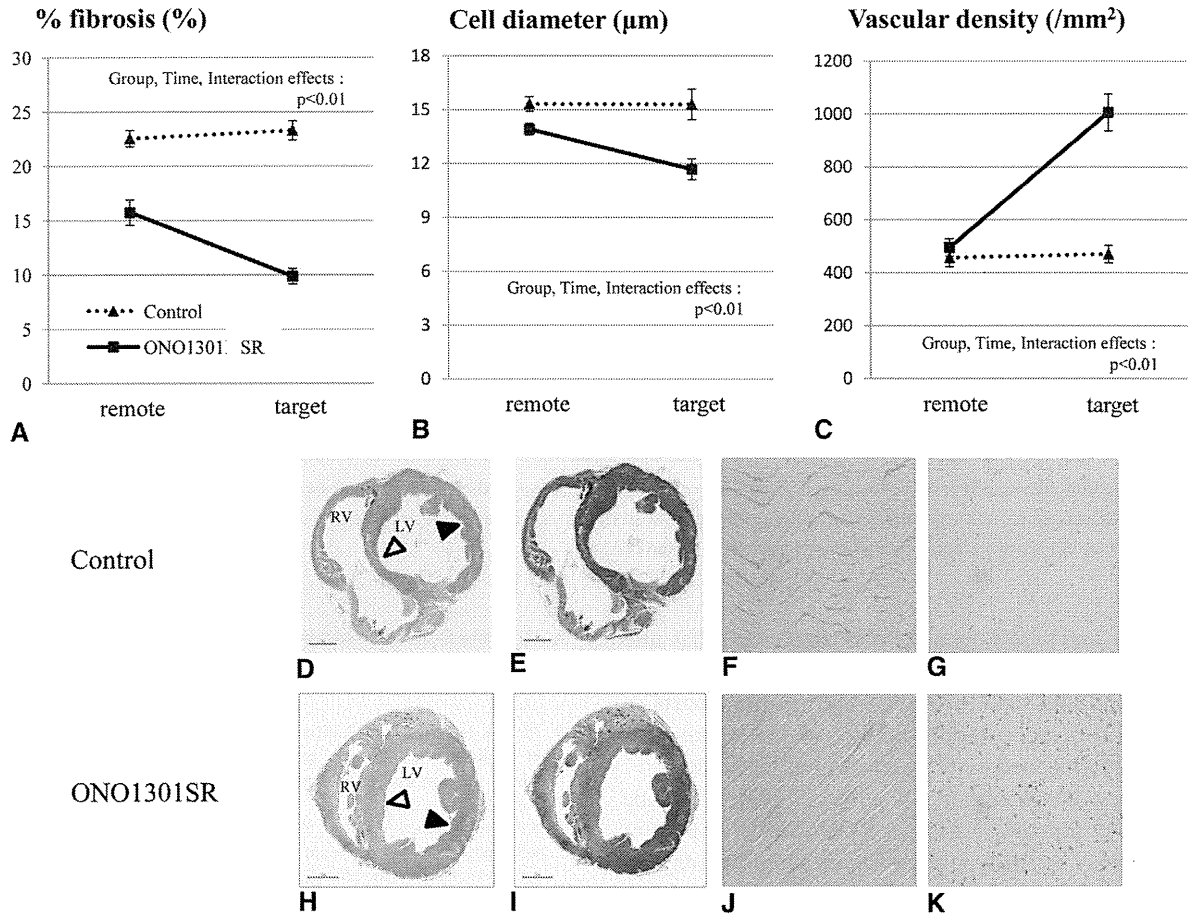

FIGURE 4. Histopathology; quantitative evaluation of interstitial fibrosis (A), mean cell diameter (B), vascular density (C), and representative micrograph of the Control group (D-G) and the ONO1301SR group (H-K). D and H, Hematoxylin and eosin staining. E and I, Masson trichrome staining. F and J, Sirius red staining. G and K, Staining with anti-human-von Willebrand factor. At the target site in the ONO1301SR group, the amounts of fibrosis and mean cell diameter were significantly smaller and vascular density was significantly higher than those in the control group and those at the remote site in the ONO1301SR group. Mean ± standard error of the mean, respectively. *LV*, Left ventricle; *RV*, right ventricle.

or attachment on the epicardial surface. Injection area–specific recovery, demonstrated in this study, would suggest that direct injection of ONO1301 might be more effective in the myocardium that has heterogeneous disease, such as ischemic cardiomyopathy, as reported by Iwata and associates.[21] Combination with coronary artery bypass grafting would also be a clinically applicable strategy for this purpose. On the other hand, homogeneous disease such as DCM might gain more therapeutic benefits by diffusely attaching ONO1301 on the epicardial surface compared with direct injection, although further basic investigation will be needed to establish this strategy. Intracoronary injection is known to diffusely deliver reagents or cells into the myocardium[25]; however, intracoronary injection of ONO1301SR whose diameter is more than 20 μm will cause coronary embolism and ischemic myocardial damage.

This study is limited by the use of a canine model, which is not exactly relevant to the clinical DCM diseases and has limited reagents for mRNA or protein investigations available.

However, a large animal model is essential in investigating cardiac performance by the latest technology used in the clinical arena, such as speckle-tracking echocardiography used in this study, whereas rodent models with or without genetic modifications would be useful in showing the mechanistic insights of this treatment. As mechanistic insights have been reported by several studies, the main focus of this study was to test the hypothesis that intramyocardial injection of ONO1301 induces region-specific and global functional recovery in dilated cardiomyopathy. In addition, this study investigated the mechanisms of this treatment to show the consistency with the previous studies that used rodent models to prove the mechanisms of this treatment.

Injection to the anterior wall and use of the posterior wall as the control was an option; however, in the surgical view, injection into the lateral wall produced consistent,

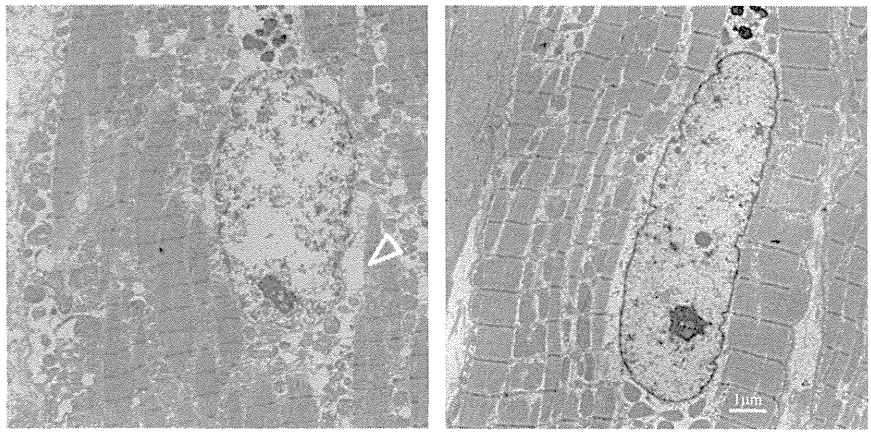

FIGURE 5. Electron microscopy revealed that the myocardium of the control group (A) showed prominent swelling or disruption of mitochondria, intracellular or perinuclear edema, and sarcoplasmic vacuoles resulting from dilation of sarcoplasmic reticulum *(arrowhead)*. On the other hand, mitochondria in the ONO1301SR group (B) were compact and showed densely packed cristae.

reproducible, and safe injections compared with that into the anterior wall. Therefore, injection of the reagent into the lateral wall and septal wall was used as the control in this study. However, pathophysiology of the septum are substantially influenced by the performance of the RV.

In summary, we quantitatively evaluated region-specific pathologic and functional effects of ONO1301SR, a slow-releasing form of prostacyclin agonist, on a rapid-pacing canine DCM model. Multitherapeutic endogenous cytokines induced by intramyocardial ONO1301SR injection may be responsible for the improved cardiac performance and ultrastructure. ONO1301SR is a promising therapeutic drug for enhancing myocardial regeneration on the impaired myocardium.

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References

1. Sharon AH, François H. The changing face of heart transplantation. *J Am Coll Cardiol*. 2008;52:587-98.
2. Maybaum S, Mancini D, Xydas S, Starling RC, Aaronson K, Pagani FD, et al. Cardiac improvement during mechanical circulatory support: a prospective multicenter study of the LVAD working group. *Circulation*. 2007;115:2497-525.
3. Cohn JN, Tognoni G, for the Valsartan Heart Failure Trial Investigators. A randomized trial of the angiotensin-receptor blocker valsartan in chronic heart failure. *N Engl J Med*. 2001;345:1667-75.
4. Packer M, Bristow MR, Cohn JN, Coluggi WS, Fowler MB, Gilbert EM, et al. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. *N Engl J Med*. 1996;334:1349-55.
5. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia–reperfusion, aging, and heart failure. *J Mol Cell Cardiol*. 2001;33:1065-89.
6. Schaper J, Froede R, Hein S, Buck A, Hashizume H, Speiser B, et al. Impairment of the myocardial ultrastructure and changes of the cytoskeleton in dilated cardiomyopathy. *Circulation*. 1991;83:504-14.
7. Unverferth DV, Baker PB, Swift SE, Chaffee R, Fetters JK, Uretsky BF, et al. Extent of myocardial fibrosis and cellular hypertrophy in dilated cardiomyopathy. *Am J Cardiol*. 1986;57:816-20.
8. Miyagawa S, Saito A, Sakaguchi T, Yoshikawa Y, Yamauchi T, Imanishi Y, et al. Impaired myocardium regeneration with skeletal cell sheets—a preclinical trial for tissue-engineered regeneration therapy. *Transplantation*. 2010;90:364-72.
9. Memon IA, Sawa Y, Fukushima N, Matsumiya G, Miyagawa S, Taketani S, et al. Repair of impaired myocardium by means of implantation of engineered autologous myoblast sheets. *J Thorac Cardiovasc Surg*. 2005;130:646-53.
10. Sitbon O, Humbert M, Nunes H, Parent F, Garcia G, Simonzeau G, et al. Long-term intravenous epoprostenol infusion in primary pulmonary hypertension: prognostic factors and survival. *J Am Coll Cardiol*. 2002;40:780-8.
11. Barst RJ, McGoon M, McLaughlin V, Tapson V, Oudiz R, Shapiro S, et al. Beraprost therapy for pulmonary arterial hypertension. *J Am Coll Cardiol*. 2003;41:2119-25.
12. Kataoka M, Nagaya N, Satoh T, Itoh T, Murakami S, Iwase T, et al. A long-acting prostacyclin agonist with thromboxane inhibitory activity for pulmonary hypertension. *Am J Respir Crit Care Med*. 2005;172:1575-80.
13. Nakamura K, Sata M, Iwata H, Sakai Y, Hirata Y, Kugiyama K, et al. A synthetic small molecule, ONO-1301, enhances endogenous growth factor expression and augments angiogenesis in the ischaemic heart. *Clin Sci (London)*. 2007;112:607-16.
14. Taniyama Y, Morishita R, Aoki M, Hiraoka K, Yamasaki K, Hashiya N, et al. Angiogenesis and antifibrotic action by hepatocyte growth factor in cardiomyopathy. *Hypertension*. 2002;40:47-53.
15. Van Belle E, Witzenbichler B, Chen D, Silver M, Chang L, Schwall R, et al. Potentiated angiogenic effect of scatter/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. *Circulation*. 1998;97:381-90.
16. Hirata Y, Soeki T, Akaike M, Sakai Y, Igarashi T, Sata M. Synthetic prostacycline agonist, ONO-1301, ameliorates left ventricular dysfunction and cardiac fibrosis in cardiomyopathic hamsters. *Biomed Pharmacol*. 2009;63:781-6.
17. Armstrong PW, Stopps TP, Ford SE, DeBold AJ. Rapid ventricular pacing in the dog: pathophysiologic studies of heart failure. *Circulation*. 1986;74:1075-84.
18. Hata H, Matsumiya G, Miyagawa S, Kondoh H, Kawaguchi N, Matsuura N, et al. Grafted skeletal myoblast sheets attenuate myocardial remodeling in pacing-induced canine heart failure model. *J Thorac Cardiovasc Surg*. 2006;132:918-24.
19. Obata H, Sakai Y, Ohnishi S, Takeshita S, Mori H, Kodama M, et al. Single injection of a sustained-release prostacyclin analog improves pulmonary hypertension in rats. *Am J Respir Crit Care Med*. 2008;177:195-201.
20. Ogawa K, Hozumi T, Sugioka K, Matsuyama Y, Nishiura M, Kanda R, et al. Usefulness of automated quantification of regional left ventricular wall motion by a novel method of two-dimensional echocardiographic tracking. *Am J Cardiol*. 2006;98:1531-7.

21. Iwata H, Nakamura K, Sumi M, Ninomiya M, Sakai Y, Hirata Y, et al. Local delivery of synthetic prostacycline agonist augments collateral growth and improves cardiac function in a swine chronic cardiac ischemia model. *Life Sci.* 2009;85:255-61.
22. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet.* 2003;362:697-703.
23. Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. *Circ. Res.* 2003;93:292-301.
24. Hazekawa M, Sakai Y, Yoshida M, Haraguchi T, Morisaki T, Uchida T. Preparation of ONO-1301-loaded poly (lactide-co-glycolide) microspheres and their effect on nerve conduction velocity. *J Pharm Pharmacol.* 2011; 63:362-8.
25. Fukushima S, Varela-Carver A, Coppen SR, Yamahara K, Felkin LE, Lee J, et al. Direct intramyocardial but not intracoronary injection of bone marrow cells induces ventricular arrhythmias in a rat chronic ischemic heart failure model. *Circulation.* 2007;115:2254-61.

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Sustained-Release Delivery of Prostacyclin Analogue Enhances Bone Marrow-Cell Recruitment and Yields Functional Benefits for Acute Myocardial Infarction in Mice

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Abstract

Background: A prostacyclin analogue, ONO-1301, is reported to upregulate beneficial proteins, including stromal cell derived factor-1 (SDF-1). We hypothesized that the sustained-release delivery of ONO-1301 would enhance SDF-1 expression in the acute myocardial infarction (MI) heart and induce bone marrow cells (BMCs) to home to the myocardium, leading to improved cardiac function in mice.

Methods and Results: ONO-1301 significantly upregulated SDF-1 secretion by fibroblasts. BMC migration was greater to ONO-1301-stimulated than unstimulated conditioned medium. This increase was diminished by treating the BMCs with a CXCR4-neutralizing antibody or CXCR4 antagonist (AMD3100). Atelocollagen sheets containing a sustained-release form of ONO-1301 (n = 33) or ONO-1301-free vehicle (n = 48) were implanted on the left ventricular (LV) anterior wall immediately after permanent left-anterior descending artery occlusion in C57BL6/N mice (male, 8-weeks-old). The SDF-1 expression in the infarct border zone was significantly elevated for 1 month in the ONO-1301-treated group. BMC accumulation in the infarcted hearts, detected by in vivo imaging after intravenous injection of labeled BMCs, was enhanced in the ONO-1301-treated hearts. This increase was inhibited by AMD3100. The accumulated BMCs differentiated into capillary structures. The survival rates and cardiac function were significantly improved in the ONO-1301-treated group (fractional area change 23±1%; n = 22) compared to the vehicle group (19±1%; n = 20; P = 0.004). LV anterior wall thinning, expansion of infarction, and fibrosis were lower in the ONO-1301-treated group.

Conclusions: Sustained-release delivery of ONO-1301 promoted BMC recruitment to the acute MI heart via SDF-1/CXCR4 signaling and restored cardiac performance, suggesting a novel mechanism for ONO-1301-mediated acute-MI heart repair.

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Competing Interests: The authors have read the journal's policy and have the following conflicts: Y. Sakai was an employee of Ono Pharmaceutical Co. Ltd., and a holder of the patent for ONO-1301 encapsulated in PLGA microspheres (patent numbers WO 2004/032965 and WO 2008/047863). There are no other patents, products in development, or modified products to declare. The other authors have declared that no competing interests exist. This does not alter the authors' adherence to all PLOS ONE policies on sharing data and materials.

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Introduction

Despite a number of medical and interventional treatments have been developed to treat acute myocardial infarction (AMI), the treatment for massive AMI has not been fully established. Myocardial infarction (MI) is a progressive disease, characterized by massive ischemic necrosis of the myocardial tissue and subsequent inflammation. This leads to cardiac remodeling that exacerbates the oxygen shortage in the surviving cardiac tissue. These pathological and functional deteriorations eventually cause end-stage heart failure. To delay the progression of heart failure, it is essential to suppress inflammation and fibrosis and to improve bloodflow supply in the injured myocardium consecutively. Recently, stromal cell-derived factor (SDF)-1 and its corresponding receptor CXCR4 have been shown to play prominent roles in homing of bone marrow cells (BMC) which promotes neovascularization and prevention of apoptosis via paracrine mechanism [1,2,3,4].

ONO-1301 (({5-[2-({[(1E)-phenyl(pyridin-3-yl)methylene]amino}oxy)ethyl]-7,8-dihydronaphthalen-1-yl}oxy)acetic acid) is a synthetic prostacyclin agonist. As it lacks the typical prostanoid

structure of a five-membered ring and an allylic alchol, ONO-1301 is chemically and biologically stable *in vivo*. In addition, thromboxane A2 synthethase is inhibited by ONO-1301, resulting in the promotion of endogenous prostacyclin synthesis. ONO-1301 has been reported to induce the production of endogenous hepatocyte growth factor (HGF) and vascular-endothelial growth factor (VEGF) in fibroblasts by stimulating cAMP production [5,6,7,8]. The administration of a slow-release form of ONO-1301 shows therapeutic potential, mainly due to the restoration of bloodflow in MI models of rat and swine and in a cardiomyopathic hamster [6,7,8]. The potential mechanism of the functional benefits of ONO-1301 mainly result from the enhanced secretion of growth factors, such as HGF and VEGF, which induce angiogenesis, restore bloodflow, and attenuate the progression of fibrosis. Recently we identified that ONO-1301 also upregulates SDF-1 secretion in the fibroblasts. Enhanced BMC homing in the MI heart by ONO-1301 therapy is attractive therapeutic modality. We thus hypothesized that ONO-1301 can induce BMC accumulation mediated by the upregulation of SDF-1 to elicit functional improvement in a mouse model of MI.

Methods

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Osaka University (H23–123). All surgery was performed under sodium pentobarbital or isoflurane anesthesia, and all efforts were made to minimize suffering.

ONO-1301 and a slow-release form of ONO-1301 were purchased from ONO Pharmaceutical Co. Ltd. (Osaka, Japan) [7,8,9].

Migration Assay

Normal human dermal fibroblasts (NHDFs; Takara bio, Shiga, Japan) were cultured with or without ONO-1301 for 72 hours. The SDF-1 concentration in the culture supernatants was measured by ELISA (R&D systems, MN). BMCs were obtained from a green fluorescent protein (GFP)-transgenic mouse [C57BL/6-Tg(CAG-EGFP); Japan SLC, Inc., Shizuoka, Japan], and their migration toward the supernatants was assessed using a culture insert system (BD Falcon). The number of migrated BMCs was determined using fluorescence microscopy (Carl Zeiss, Göttingen, Germany).

Mouse AMI Model and Sheet Transplantation

An AMI model was generated by permanent ligation of the left anterior descending artery (LAD) in 10-15-week-old male C57BL/6N, BALB/cA, or BM-GFP chimera mice [10]. ONO-1301 microspheres and control microspheres were resuspended in saline at 10 mg/ml and added to atelocollagen sheets just before transplantation. Five minutes after the LAD ligation, atelocollagen sheets that included ONO-1301-containing microspheres (ONO-1301-treated group, n = 40) or empty microspheres (vehicle group, n = 40) were fixed onto the surface of the anterior left ventricular (LV) wall. The mice were euthanized 7, 21, and 28 days after the LAD ligation and ONO-1301 administration.

Assessment of BMC Homing

BMCs harvested from BALB/cA mice were labeled by Xenolight DiR (Caliper Life Sciences, MA) following the manufacturer's instructions and injected into the tail vein of BALB/cA mice after the MI and ONO-1301 treatment. On days 1 and 3, the whole-body imaging of the mice was measured by an *in vivo* imaging system (IVIS, Caliper Life Sciences).

Assessment of Cardiac Function and Survival

Cardiac function was assessed using an echocardiography system equipped with a 12-MHz transducer (GE Healthcare, WI) 4 weeks after MI and ONO-1301 treatment. The LV areas were measured, and LV fractional area change (FAC) was calculated as (LVEDA-LVESA)/LVEDA×100, where LVEDA and LVESA are the LV end-diastolic and end-systolic area, respectively.[10] The mice were housed in a temperature-controlled incubator for 28 days post-treatment to determine their survival.

Histological Analysis

Frozen sections (8 μm) of hearts were stained with antibodies against von Willebrand factor (vWF; Dako, Glostrup, Denmark) and CD31 (Abcam, UK). The secondary antibody was Alexa 546 goat anti-rabbit (Life Technologies, CA). Counterstaining was performed with 6-diamidino-2-phenylindole (DAPI; Life Technologies). The sections were also stained with isolectin (Life Technologies) following the manufacturer's instructions. To count GFP-positive cells, isolectin-positive cells, and CD31-positive capillary densities, 10 images were captured for each specimen. Capture and analysis were performed using Biorevo (Keyence, Japan). To analyze the myocardial collagen accumulation, heart sections were stained with Masson's trichrome. The collagen volume fraction in the peri-infarct area was calculated.

Quantitative Real-time PCR

The total RNA was isolated from the peri-infarct area using the RNeasy Mini Kit and reverse transcribed using Omniscript Reverse transcriptase (Qiagen, Hilden, Germany). Quantitative PCR was performed with a PCR System (Life Technologies). The expression of each mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers and probes are shown in Table S1 in File S1.

Statistical Analysis

Data are expressed as the mean ± SEM. The data distributions were checked for normality. Comparisons between 2 groups were made using the Student's *t*-test. For comparisons among 3 or more groups, one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test were used. The survival curves were prepared using the Kaplan-Meier method and compared using the log-rank test. All *P*-values are two-sided, and values of $P<0.05$ were considered to indicate statistical significance. Statistical analyses were performed using the StatView 5.0 Program (Abacus Concepts, Berkeley, CA) and Statcel2 (The Publisher OMS Ltd., Saitama, Japan).

An expanded Methods section can be found in the online-only in File S1.

Results

ONO-1301 Enhanced BMC Migration via SDF-1/CXCR4 Signaling

The effect of ONO-1301 on the SDF-1 secretion by NHDFs was evaluated by ELISA. As shown in Fig. 1A, the SDF-1 concentration in the NHDF culture supernatants increased in an ONO-1301 concentration-dependent manner. The SDF-1 concentration in the culture supernatant of 1000 nM ONO-1301-treated cells was significantly greater than that of cells cultured in

the absence of ONO-1301 (Fig. 1A). To investigate the BMC migration toward ONO-1301-treated NHDF conditioned medium, a migration assay was performed using a modified Boyden chamber with 8-μm pores. The number of migrated BMCs was significantly greater in the conditioned medium of cells treated with 100 and 1000 nM ONO-1301 compared to that of cells treated with 0 and 10 nM ONO-1301. The BMC migration to the 1000 nM ONO-1301 conditioned medium was diminished by treating the BMCs with a CXCR4-neutralizing antibody or CXCR4 antagonist (AMD3100) (Fig. 1B, C).

SDF-1-mediated BMC Accumulation in the ONO-1301-treated Infarcted Hearts

The effect of ONO-1301 on SDF-1 expression in the infarcted hearts was evaluated by quantitative RT-PCR. Twenty-eight days after treatment, the SDF-1 expression in the border area of the ONO-1301-treated heart was significantly greater than that in the vehicle-treated heart (Fig. 2A). The HGF and VEGF expressions were also increased by ONO-1301 treatment (Fig. 2B, C). After LAD occlusion, ONO-1301 treatment, and intravenous injection of labeled BMCs, the BMC accumulation in the infarcted heart was evaluated by an in vivo imaging system. The proportion of BMCs in the heart showed a trend toward upregulation, dependent on the dose of ONO-1301 (Fig. 2D). Hearts treated with 100 mg ONO-1301/kg body weight showed significantly more accumulated BMCs than those treated with 0 or 10 mg ONO-1301. In 100 mg/kg ONO-1301-treated hearts, CXCR4 antagonization significantly decreased the BMC accumulation (Fig. 2D). To identify the recruited BMCs *in vivo*, the acute MI model was prepared using chimera mice by transplanting GFP-expressing bone marrow into irradiated C57BL/6 mice. The BMCs of the C57BL/6 transplant recipients were largely replaced by GFP-expressing BMCs (91.8+/−4.3%, figure S1 in File S1). The single-organ analyses using GFP-BM chimera mouse at day 7 also showed increased BMC accumulation in the ONO-1301-treated myocardium (figure S2 in File S1).

Differentiation of BMCs in the Infarcted Myocardium

Seven days after MI and ONO-1301 administration to BM-GFP chimera mouse, BMCs were dramatically accumulated in both the infarcted area and the atelocollagen sheet (Fig. 3A, B). Some of the BMCs formed tube-like structures and displayed von Willebrand factor expression (Fig. 3C, D). Isolectin staining showed that a greater percentage of isolectin-positive BMCs accumulated in the myocardium in the ONO-1301-treated (O) group than in the vehicle (V) group (Fig. 3E, F). We also evaluated small blood vessels by CD31 immunostaining. The density of small vessels was greater in the O group than in the V group (Fig. 3G). Immunohistochemical analysis of Connexin 43 and smooth muscle actin, cardiac-lineage and cardiac fibroblast markers, respectively, was also conducted at 3 months, but no co-expression

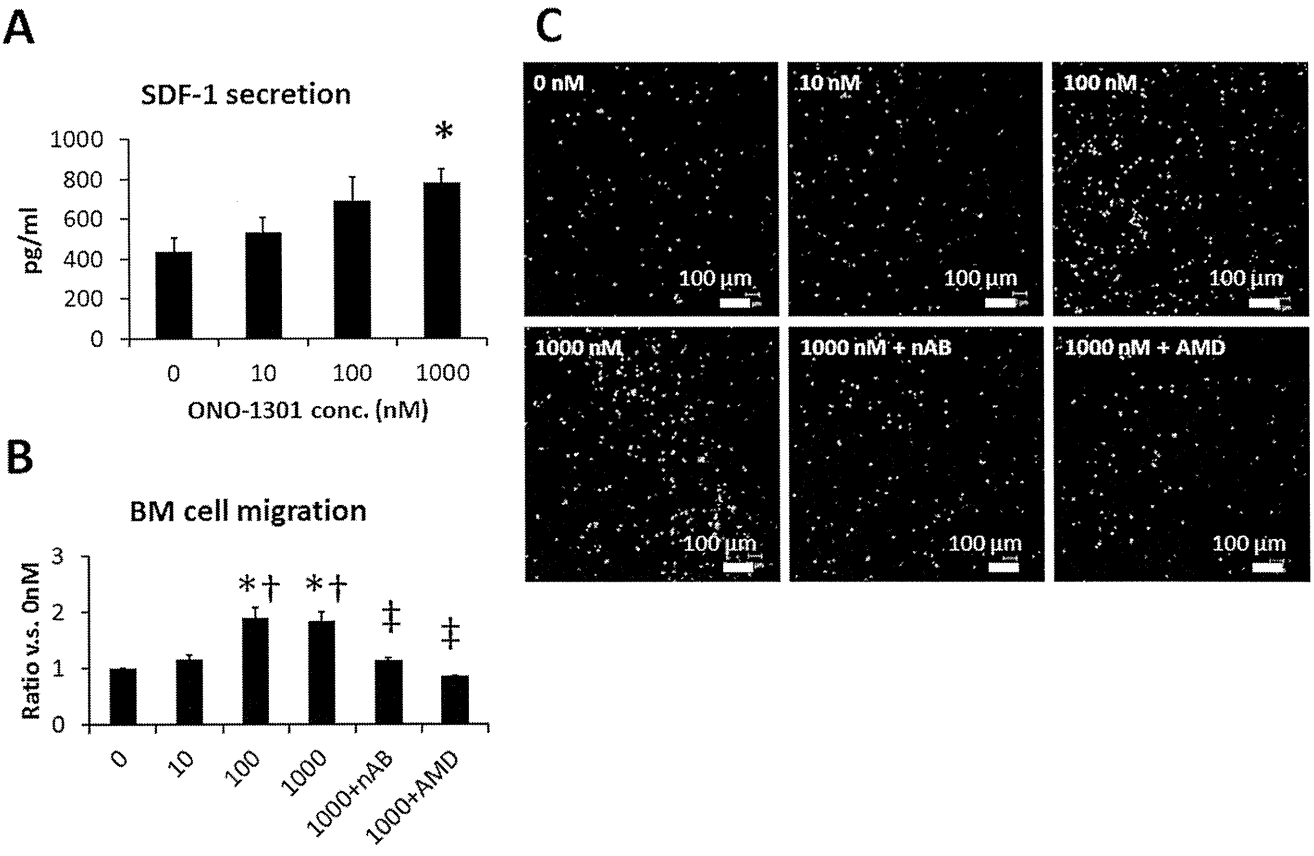

Figure 1. ONO-1301 enhanced SDF-1 secretion and BMC migration via SDF-1/CXCR4 signaling *in vitro*. NHDFs were stimulated with ONO-1301 for 72 hours, then the SDF-1 concentration in the culture medium was determined by ELISA (n = 3 each, *P<0.05 vs. 0 nM). A) Number of BMCs that migrated toward the conditioned medium from ONO-1301-stimulated-NHDFs (0, 10, 100, or 1000 nM ONO-1301, n = 6; 1000 nM+nAB or 1000 nM+AMD, n = 3). *P<0.05 vs. 0 nM, †P<0.05 vs. 10 nM, ‡P<0.05 vs. 1000 nM, §P<0.05 vs. SDF-1. nAB, CXCR4-neutralizing antibody; AMD, CXCR4 antagonist AMD3100. B) Representative pictures of BMCs that had migrated to the medium from ONO-1301-stimulated BMCs. Green, BMCs.
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