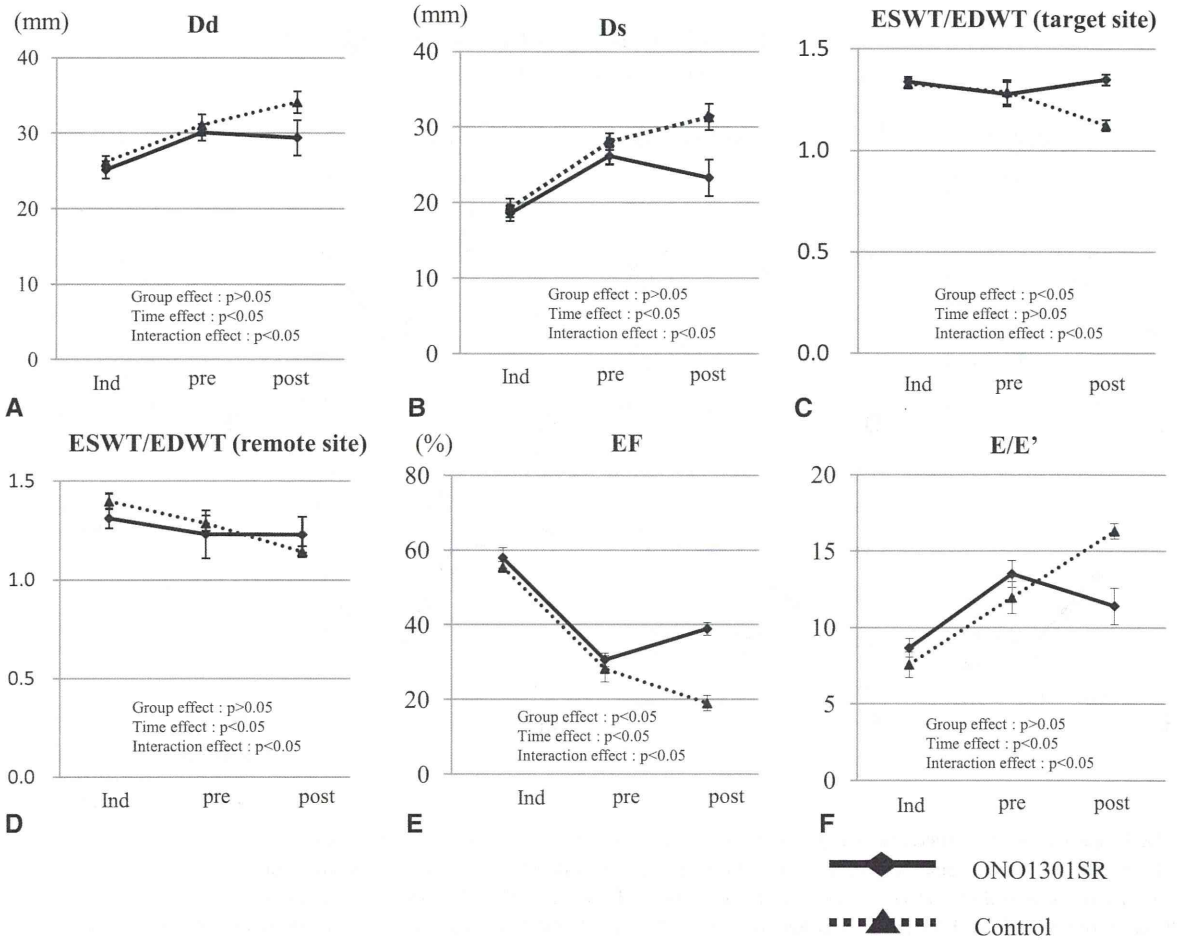

FIGURE 2. Echocardiography (A-F) showed ONO1301SR significantly improved distressed cardiac function. Note that ESWT/EDWT, reflecting on the radial strain of myocardium, was significantly more recovered in the ONO1301SR group than in the control group and that the clinical impact was more prominent in 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

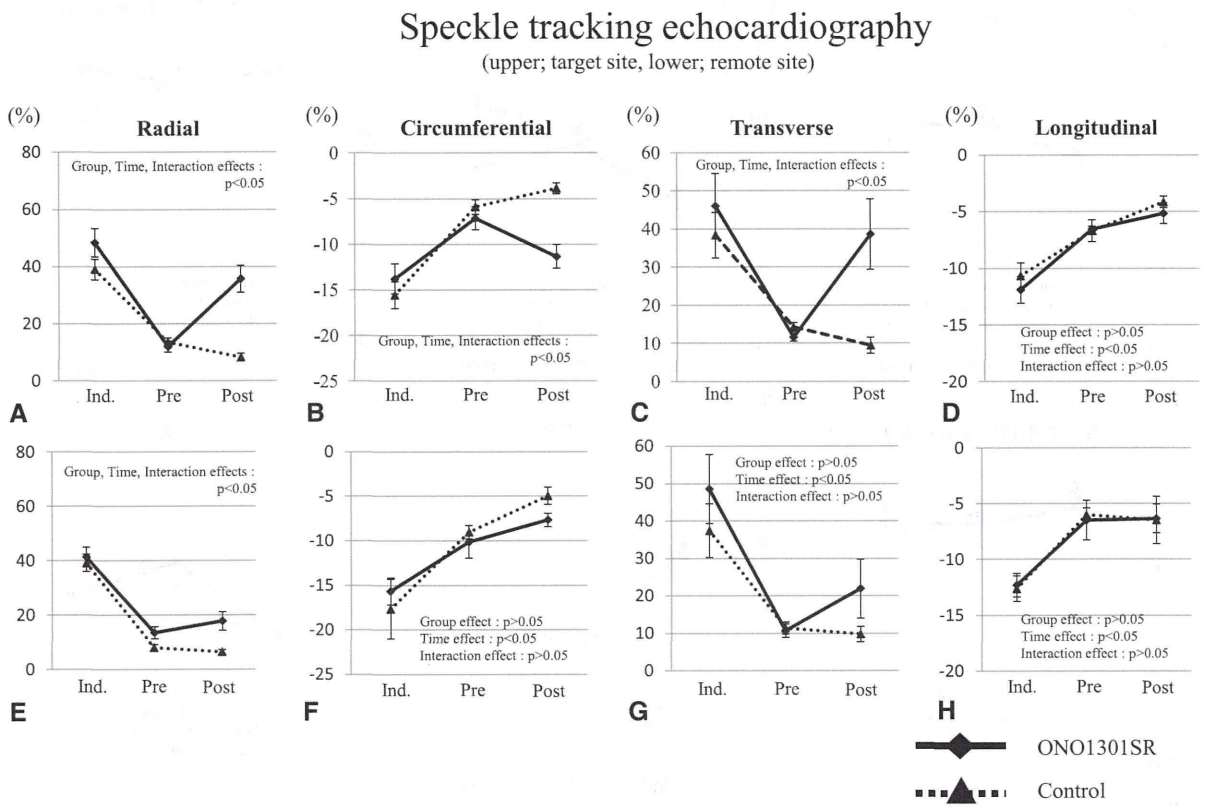

FIGURE 3. Speckle-tracking echocardiography showed that the absolute values of peak radial, circumferential, and transverse strains at the target site in the ONO1301SR group were significantly higher than in the control group (A-D) while all of them but radial strains at the remote site in the ONO1301SR 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.

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,

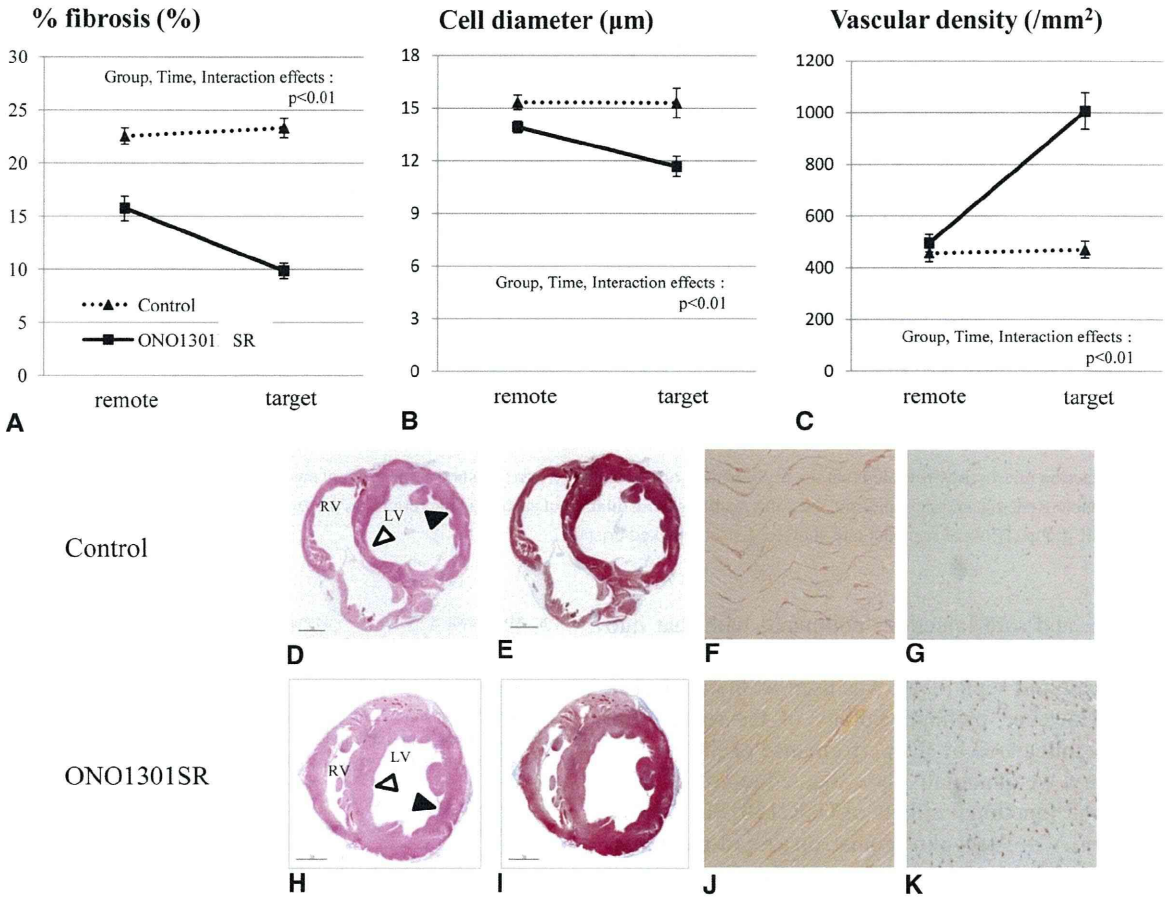

FIGURE 4. Histopathology; quantitative evaluation of interstitial fibrosis (A), mean cell diameter (B), vascular density (C), and representative micrograph of the Control group (D-G) and the ONO1301SR group (H-K). D and H, Hematoxylin and eosin staining. E and I, Masson trichrome staining. F and J, Sirius 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,

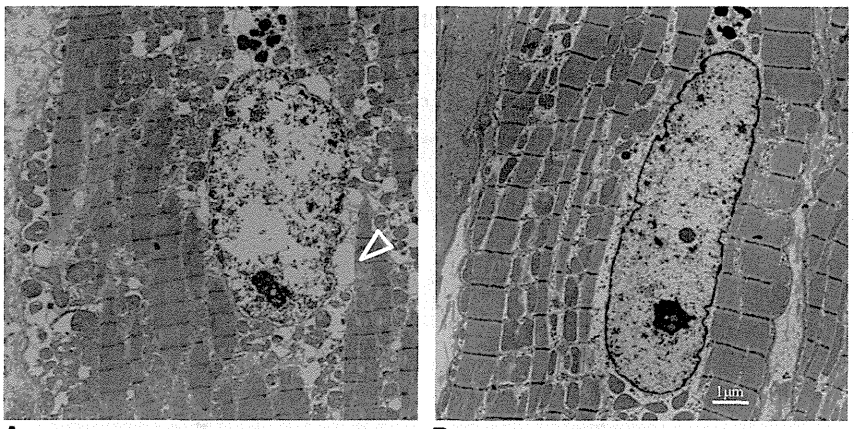

FIGURE 5. Electron microscopy revealed that the myocardium of the control group (A) showed prominent swelling or disruption of mitochondria, intracellular or perinuclear edema, and sarcoplasmic vacuoles resulting from dilation of sarcoplasmic reticulum *(arrowhead)*. On the other hand, mitochondria in 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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Synthetic prostacyclin agonist, ONO1301, enhances endogenous myocardial repair in a hamster model of dilated cardiomyopathy: A promising regenerative therapy for the failing heart

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Objectives: Remodeling of the left ventricle (LV) in idiopathic dilated cardiomyopathy (IDCM) is known to be associated with multiple pathologic changes that endogenous factors, such as hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF), protect against. Although a clinically relevant delivery method of these factors has not been established, ONO1301, a synthetic prostacyclin agonist, has been shown to upregulate multiple cardioprotective factors, including HGF and VEGF, in vivo. We thus hypothesized that ONO1301 may reverse LV remodeling in the DCM heart.

Methods: ONO1301 dose-dependently added to the normal human dermal fibroblasts and human coronary artery smooth muscle cells in vitro, to measure the expression of HGF, VEGF, stromal cell–derived factor (SDF)-1, and granulocyte-colony stimulating factor (G-CSF), assessed by real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay. δ-Sarcoglycan-deficient J2N-k hamsters, which is an established DCM model, were treated by epicardial implantation of an atelocollagen sheet with or without ONO1301 immersion or sham operation.

Results: ONO1301 dose-dependently upregulated expression of these 4 factors in vitro. ONO1301 treatment, which induced dominant elevation of ONO1301 levels for 2 weeks, significantly preserved cardiac performance and prolonged survival compared with the other groups. This treatment significantly upregulated expressions of cardioprotective factors and was associated with increased capillaries, attenuated fibrosis, and upregulation of α-sarcoglycan in the DCM heart.

Conclusions: ONO1301 atelocollagen-sheet implantation reorganized cytoskeletal proteins, such as α-sarcoglycan, increased capillaries, reduced fibrosis, and was associated with upregulated expression of multiple cardioprotective factors, leading to preservation of cardiac performance and prolongation of survival in the δ-sarcoglycan-deficient DCM hamster. (J Thorac Cardiovasc Surg 2013;146:1516-25)

Idiopathic dilated cardiomyopathy (IDCM) is one of the most critical intractable diseases. The etiology and pathology of IDCM have therefore been intensively investigated to explore other treatment options.[1] Clinical and functional progression of IDCM has been shown to be closely correlated with the histopathology, such as apoptosis of cardiomyocytes, accumulation of fibrotic components, reduction of vascular density, and remodeling of sarcolemmal/cytoskeletal proteins. It has been recently suggested that cell transplantation into the IDCM heart positively modulates cellular behavior of native cardiac fibroblasts and/or coronary artery smooth muscle cells (CoASMCs), leading to upregulation of multiple cardioprotective factors in the heart.[2] Inasmuch as cell transplantation is clinically limited by the cell-culture procedure and the availability of a cell processing center, cell-free therapy that enhances cardiac regeneration has long been sought in the clinical arena.[3]

Prostacyclin and its analogs have been shown to upregulate expressions of various factors, such as hepatic growth factor (HGF) and vascular endothelial growth factor (VEGF) in vitro and in vivo.[4] Although previously generated prostacyclin agonists are chemically unstable, being limited by the delivery method, it has recently been shown that ONO1301 is a selective prostacyclin receptor (IPR) agonist having a unique, chemically stable structure, and polymerization of ONO1301 with polylactic-co-glycolic acid copolymer (PLGA) to form a microsphere (ONO1301-MS) upregulates multiple protective factors, represented by HGF, for 3 to 4 weeks in vivo.[5] We therefore

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Abbreviations and Acronyms

CoASMC = coronary artery smooth muscle cell
DCM = dilated cardiomyopathy
Dd/Ds = diastolic/systolic dimensions
EF = ejection fraction
ELISA = enzyme-linked immunosorbent assay
GAPDH = glyceraldehyde-3-phosphate dehydrogenase
G-CSF = granulocyte colony stimulating factor
HCoASMC = human coronary artery smooth muscle cell
HGF = hepatic growth factor
IPR = prostacyclin receptor
IDCM = idiopathic dilated cardiomyopathy
LV = left ventricular (ventricle)
N group = atelocollagen sheet without ONO1301
NHDF = normal human dermal fibroblast
O group = atelocollagen sheet containing ONO1301
PCR = polymerase chain reaction
PLGA = polylactic-co-glycolic acid copolymer
S group = sham group
SDF-1 = stromal cell–derived factor-1
VEGF = vascular endothelial growth factor
vWF = von Willebrand factor

hypothesized that administration of ONO1301-MS into the IDCM heart might upregulate cardiac protective factors, leading to histologic and functional reverse left ventricular (LV) remodeling.

MATERIALS AND METHODS

Experimental procedures related to animal studies were carried out under the approval of the institutional ethics committee. The investigation conformed to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health Publication No. 85 to 23, revised 1996). All experimental procedures and evaluations were performed in a blinded manner.

Cell Culture

Normal human dermal fibroblasts (NHDFs) and human CoASMCs (HCoASMCs) were purchased from EIDIA Co, Ltd (Tokyo, Japan). The cells were cultured on 6-well plates with Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, Mo) supplemented with 10% fetal bovine serum (EIDIA) under 5% carbon dioxide. Next, 1- to 1000-nmol/L ONO1301 (Ono Pharmaceutical, Osaka, Japan), dimethyl sulphoxide (Sigma-Aldrich) or dibutyryl cyclic adenosine monophosphate (Sigma-Aldrich) was added to the culture medium for 72 hours, and then the culture supernatants and cells (n = 6, respectively) were harvested and stored at −80°C.

Procedure of ONO1301-MS Administration to the Dilated Cardiomyopathy (DCM) Hamster

Male 20-week-old δ-sarcoglycan-deficient J2N-k hamsters and J2N-n normal hamsters were purchased from Japan SLC (Shizuoka, Japan). Inasmuch as human DCM-like histopathologic features and associated functional deterioration develop in J2N-k hamsters, they have been used as an established IDCM model.[6] Each hamster underwent left lateral thoracotomy under 1.5% isoflurane anesthesia (n = 66). Subsequently, ONO-1301 was delivered into the heart using a novel drug delivery system, in which an atelocollagen sheet (Integran sheet; Koken Co, Ltd, Tokyo, Japan) shaped "hand-drum" containing ONO1301-MS (10 mg/kg) was placed to cover the entire ventricular free wall (O group, n = 22). Other hamsters underwent either ONO1301-free atelocollagen sheet implantation in the same manner (N group, n = 21) or sham operation (S group, n = 23). After the layered closure, the hamsters were housed in a temperature-controlled individual cage until spontaneous or scheduled death at 2 or 4 weeks after the operation (n = 5 each).

Measurement of ONO1301 Concentration in the Plasma and the Ventricular Tissue

Under isoflurane inhalation (5%), venous blood (1 mL) was sampled from the internal jugular vein, and the ventricle was then excised from the hamster at day 1 and weeks 1, 2, 4, and 8 after ONO1301 treatment (n = 3 each). The plasma was stored at −80°C, and the ventricle was thoroughly washed and stored at −80°C. The concentrations of ONO1301 in the plasma and the ventricle were measured by high-performance liquid chromatography with the tandem mass spectrometric (LC/MS/MS) detection.[7]

Transthoracic Echocardiography

Transthoracic echocardiography was performed using a system equipped with a 12-MHz transducer and SONOS 5500 (Agilent Technologies, Palo Alto, Calif) under isoflurane inhalation (1%). Diastolic/systolic dimensions (Dd/Ds) and ejection fraction (EF) of the LV were measured.[8]

Histopathology

The heart was excised under isoflurane anesthesia (5%) and immersion-fixed with ice cold 4% paraformaldehyde. The fixed heart was embedded with either paraffin or optimal cutting temperature compound (Funakoshi, Tokyo, Japan) and transversely sliced to generate paraffin or frozen sections, respectively. The paraffin sections were stained using picrosirius red or immunohistologically labeled using anti–von Willebrand factor (vWF) antibody (DAKO, Glostrup, Denmark). Frozen sections (7-μm thick) were immunohistologically labeled using anti–α-dystroglycan (clone: VIA4-1; Upstate Biotechnology, Lake Placid, NY), anti–α-sarcoglycan (clone: Ad1/20A6; Novocastra, Wetzlar, Germany), anti–β-sarcoglycan (clone: bSarc/5B1; Novocastra), anti-IPR (Abcam, Cambridge, United Kingdom), or anti–α-actin (Millipore, Billerica, Mass) antibodies. The sections were then labeled by corresponding AlexaFluor488/594-conjugated secondary antibodies counterstained with 6-diamidino-2-phenylindole (DAPI; Life Technologies, Calif). 3,3′-Diaminobenzidine (DAB) staining of IPR was performed using the LSAB2 kit (DAKO). Fluorescent-labeled sections were viewed under an ECLIPSE TE 200-U confocal microscope (Nikon, Tokyo, Japan). The percentage of the total area that was fibrotic, as determined by picrosirius red staining, was calculated by using a planimetric method with MetaMorph software (Molecular Device, Osaka, Japan). The number of capillaries per square millimeter was calculated by the BZ Analyzer (Keyence, Osaka, Japan) and was counted in 4 high-power fields per section (a total of 10-12 fields/heart).

Real-Time Polymerase Chain Reaction

Total RNA was isolated from the cultured cells and the free wall of the LV using the RNeasy Kit and reverse-transcribed using Omniscript Reverse

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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

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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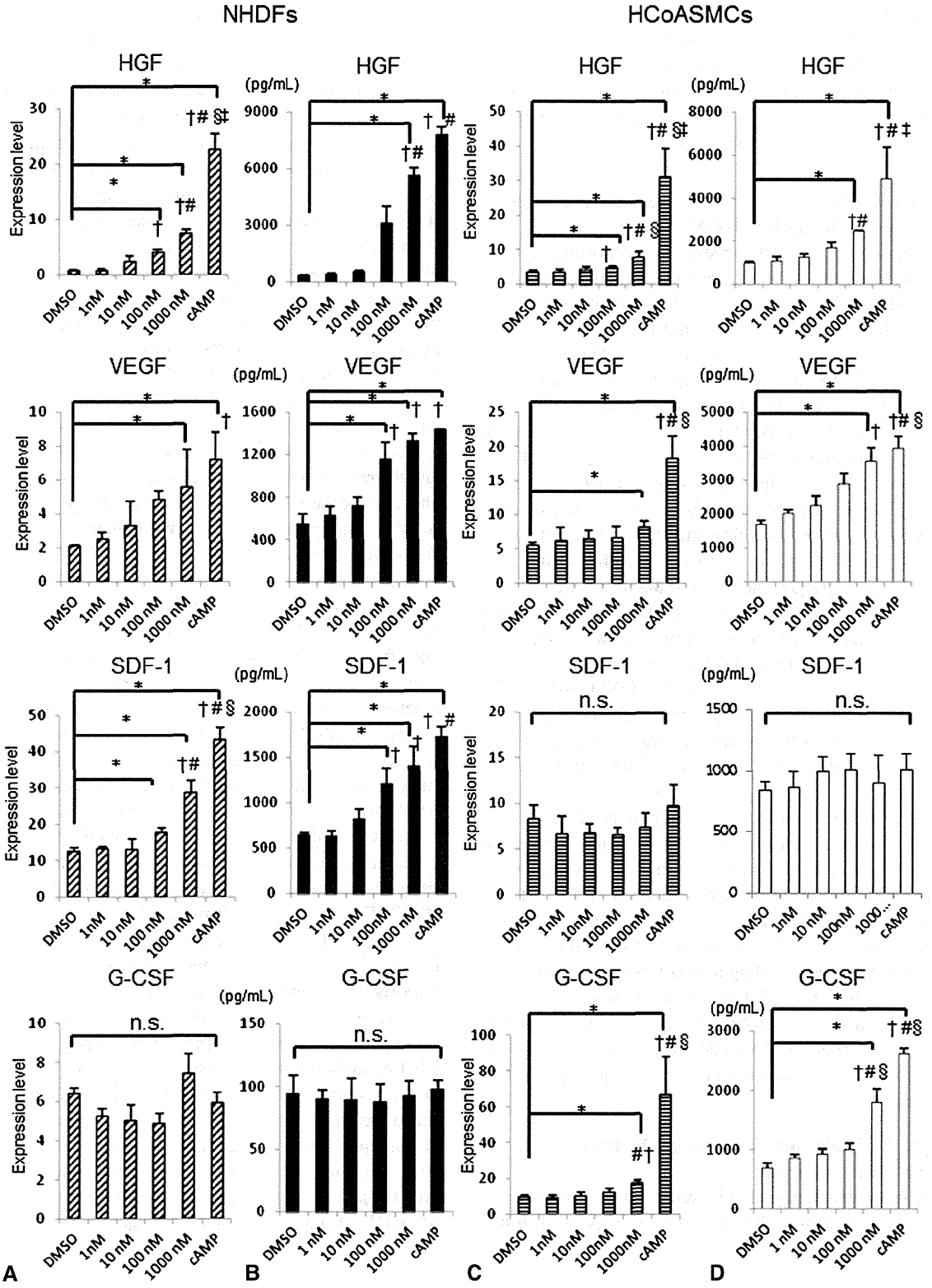

FIGURE 1. Effects of ONO1301 on the production of cardiac protective factors in NHDFs and HCoASMCs were assessed in vitro. ONO1301 treatment significantly upregulates expressions of HGF, VEGF, and SDF-1 in NHDFs, assessed by real-time PCR (A). HGF, VEGF, and SDF-1, but not G-CSF, are significantly secreted 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);