厚生労働科学研究費補助金（難治性疾患等克服研究事業(難治性疾患克服研究事業)）

分担研究報告書

ONO-1301の新規ナノスフェアー製剤の作製と有効性の確認

研究分担者　　大阪大学医学部附属病院　　講師　齋藤　充弘
統計担当：兵庫医科大学医学部　　准教授 大門　貴志

研究要旨

疾患特異的なDDSナノスフェアー製剤として、新規ONO-1301ナノスフェアー製剤（ONO-1301NS）を作製し、間歇静脈内投与により、より安全で汎用性の高い疾患特異的（DDS）な重症心不全治療剤開発を目的とした治療法の開発を検討した（特許出願準備中）。

特性の異なる代表的な２種のONO-1301NS製剤を作製し、本製剤を重症心不全（肺高血圧症）モデルに間歇静脈内投与し、ONO-1301と比較した。即ち、ラットにモノクロタリン60 mg/kgを単回皮下投与により誘発させた重症心不全（肺高血圧症）モデルを用いて、モデル作製７日後より、ONO-1301；３ mg/kg x ２回／日反復経口投与、及び ONO-1301 又は特性の異なる２種の ONO-1301NS を週１回、（ONO-1301として）１mg/kg間歇静脈内投与し、４２日までの生存率を比較検討し、ONO-1301NS製剤のDDS効果を確認した。

その結果、媒体投与群（Cont）に比し、ONO-1301反復経口投与群およびONO-1301NS製剤（A）の間歇静注投与群は共に５０%の有意な生存率の延長を示した。このことから、ONO-1301 の総投与量は NS剤にすることにより1／42与量で同等の効果を示すことから、ONO-1301のNS製剤化により間歇静脈内投与により ONO-1301 が疾患局所に集積（DDS）され、より少量投与により有効性を発揮する可能性が示唆された。ONO-1301NS製剤は、安全性、汎用性、利便性、経済性に優れたDDS製剤になることが示唆された。

A．研究目的

拡張型心筋症対する治療法としては、重症拡張型心筋症患者へは補助人工心臓装着時に人工心臓離脱を目的として、ONO-1301MSシート製剤を心臓貼付投与することを目的として検討している。

一方、軽症・中等症患者に対しては、ONO-1301反復経口投与により心臓移植や人工心臓の装着を遅らせたり、回避することを目的として検討している。

しかし、心臓貼付における開胸手術は患者への侵襲が大きく、汎用性は少ない。一方、経口投与は全身投与であるため、心臓特異性は低く、安全性との乖離が危惧される。

今回、間歇反復静脈内投与による疾患局所特異的な製剤として ONO-1301 ナノスフェアー製剤を作製し、安全性、汎用性、利便性、経済性を目指した新製剤の作製とその有用性を検討することを目的とした。

B．研究方法

疾患特異的な DDS（Drug Delivery System）ナノスフェアー製剤として、特性の異なる代表的な２種の新規ONO-1301ナノスフェアー製剤（ONO-1301NS製剤A及びB）を作製し、間歇反復静脈内投与により、より安全で汎用性の高い疾患局所特異的（DDS）な重症心不全治療剤開発を目的とした治療法の検討を行った。

代表的な２種のONO-1301NS製剤（ONO-1301NS製剤（A）；平均粒子径122nm及びONO-1301NS製剤（B）；平均粒子径；109nm）を作製した（特許出願準備中のため詳細記載略）。

作製したONO-1301NS製剤をラットモノクロタリン(MCT)誘発重症心不全（肺高血圧症）モデルに週１回間歇静脈内投与し、ONO-1301反復経口投与と比較し、有用性を検討した。

１．　被験物質及び媒体

１）ONO-1301
- Lot No.：H5001（小野薬品より入手）

２）ONO-1301NS剤A
- Lot No.：D1441
- 平均粒子径：122nm

３）ONO-1301PLNS剤B
- Lot No.：YT140118
- 平均粒子径：109 nm

４）陽性対照（ET-1拮抗剤）；ボセンタン
- Lot No.：0701012325b

５）媒体

（１）　0.5 %カルボキシメチルセルロース水溶液（以下、0.5% CMC-Na水溶液）
- Lot No. WAG1264、和光純薬工業㈱

（２）　注射用水
- Lot No. 3D86N、日本薬局方、㈱大塚製薬

２．　試験系

１）使用動物

動物種：ラット

系統： Slc:Wistar（雄性）

入荷時週齢　　：４週齢（試験２）
試験開始時週齢：５週齢（試験２）
入荷時体重　　：66.4～90.8 g
供給源　　　　：日本エスエルシー株式会社

３．試験方法

１）重症心不全（肺高血圧）モデル誘発物質

名称　　　：モノクロタリン（以下、MCT）
Lot No.　 ：SLBG1999V
製造元　　：Sigma-Aldrich Corporation

２）重症心不全モデルの作製（肺高血圧症モデル作製）

MCT を 60 mg//kg の用量で単回背部皮下投与し、MCT 投与６日後に体重層別割付法で群分けした。

３）群構成

群	投与物質、投与用量、投与回数	投与経路	例数
1	生理食塩液/週	静脈内	20
2	ONO-1301 ; 3 mg/kg×2 回/日	経口	10
3	ボセンタン ; 50 mg/kg×2 回/日	経口	10
4	ONO-1301NS 剤 A ; 1 mg/kg/週	静注	10
5	ONO-1301NS 剤 B ; 1 mg/kg/週	静脈内	10
6	ONO-1301 ; 1 mg/kg/週	静脈内	10

＊投与量は ONO-1301 としての用量を示す。

１群：MCT 投与 7、14、21、28 および 35 日後（計５回）に１週間間隔で生理食塩液を静脈内投与した。
２群：MCT 投与後 7～41 日後まで、投与の間隔を８時間以上空けて、ONO-1301 の 3 mg/kg を２回/日、経口投与した。
３群：MCT 投与 7～41 日後まで、投与の間隔を８時間以上空けて、ボセンタンの 50 mg/kg を２回/日、経口投与した。
４群：MCT 投与 7、14、21、28 および 35 日後（計５回）に１週間間隔で ONO-1301NS 剤 A の 1 mg/kg を静脈内投与した。
５群：MCT 投与 7、14、21、28 および 35 日後（計５回）に１週間間隔で ONO-1301NS 剤 B の 1 mg/kg を静脈内投与した。
６群：MCT 投与 7、14、21、28 および 35 日後（計５回）に１週間間隔で ONO-1301 の 1 mg/kg を静脈内投与した。

４）評価

（１）一般状態観察

MCT 投与 7～41 日後までは８時間以上間隔を空けて２回/日、動物の瀕死状態を確認した。尚、飼育期間中に瀕死状態（耳介反射、聴覚反射、痛覚反射の検査において１項目でも反応しない場合）をもって死亡と判断した。

（２）体重測定

MCT 投与日の投与前、MCT 投与３および６（群分け日）日後に測定した。MCT 投与 7、9、12、15、18、21、24、27、30、33、36 および 39 日後は１回目の被験物質投与前に測定した。MCT 投与 42 日後は午前中に測定した。

（倫理面への配慮）

前臨床・非臨床研究においては、各種法令・告示・通知に基づき研究を実施する。加えて、臨床試験の実施に際しては、研究計画書に関して倫理委員会での承認を受け、将来的には治験を行い、最終的には薬事申請を行う。

Ｃ．研究結果

１．生存率

重症心不全モデル作製 42 日後までの生存率の推移を Figure. 1、および Table１に示す。

対照群（1 群）は、MCT の 60 mg/kg 皮下投与により重症心不全モデル作製 15 日後に 1 例の死亡が観察された。その後、42 日までに 17 例の死亡が観察され、最終生存率は 10 %（生存数：2/20 例）であった。

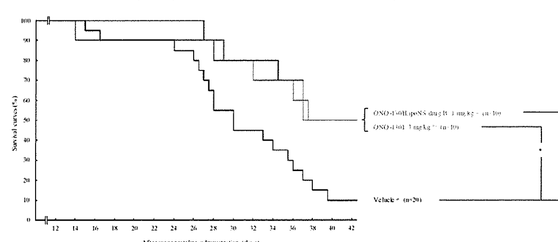
ONO-1301 の 3 mg/kg の２回/日、反復経口投与群（２群）は、重症心不全モデル作製 14 日後に 1 例の死亡が観察された。その後、42 日までに 4 例の死亡が観察され、最終生存率は 50 %（生存数：5/10 例）であり、対照群（1 群）と比較して有意な延命効果が認められた（p<0.05）。

ボセンタンの 50 mg/kg の２回/日、反復経口投与群（3 群）は、重症心不全モデル作製 20 日後に 1 例の死亡が観察された。その後、42 日までに 6 例の死亡が観察され、最終生存率は 30 %（生存数：3/10 例）であり、対照群（1 群）と比較して有意な延命効果は認められなかった。

ONO-1301NS 剤 A の 1 mg/kg の１回/週、間歇静脈内投与群（4 群）は、重症心不全モデル作製 27 日後に 1 例の死亡が観察された。その後、42 日までに 4 例の死亡が観察され、最終生存率は 50 %（生存数：5/10 例）であり、対照群（1 群）と比較して有意な延命効果が認められた（p<0.05）。

ONO-1301NS 剤 B の 1 mg/kg の１回/週、間歇静脈内投与群（5 群）は、重症心不全モデル作製 18 日後に 1 例の死亡が観察された。その後、42 日までに 8 例の死亡が観察され、最終生存率は 10 %（生存数：1/10 例）であり、対照群（1 群）と比較して有意な延命効果は認められなかった。

ONO-1301 の 1 mg/kg の 1 回/週、間歇静脈内投与群（6 群）は、重症心不全モデル作製 28 日後の投与約 30 分後に 2 例の死亡が観察された。その後、42 日までに 6 例の死亡が観察され、最終生存率は 20 %（生存数：2/10 例）であり、対照群（1 群）と比較して有意な延命効果は認められなかった。



・投与量は ONO-1301 としての用量を示す。
＊：P ＜ 0.05　vs１群

Fig１：ONO-1301 反復経口投与群と ONO-1301NS 製剤（A）の間歇静脈内投与における 42 日間の生存曲線

群	投与物質、投与量、投与回数	投与経路	例数	生存率（%）
1	生理食塩水	静注	２０	１０
2	ONO-1301;3mg/kg x ２回/日	経口	１０	５０*
3	ﾎﾞｾﾝﾀﾝ；50mg/kg x ２回/日	経口	１０	３０
4	ONO-1301NS 剤Ａ； 1 mg/kg/週	静注	１０	５０*
5	ONO-1301NS 剤Ｂ； 1 mg/kg/週	静注	１０	１０
6	ONO-1301 ; 1mg/kg/週	静注	１０	２０

Table１：各群４２日後の生存率

２）体重

MCTの60 mg/kg投与時の各群の平均体重は115.5～119.1 gを示し、被験物質投与開始時（MCT投与７日後）の各群の平均体重は137.4～139.6 gであった。

対照群（1群）はMCT投与後、平均体重は増加して21日後は188.2 g（18/20例）であった。その後、体重が減少し16例の死亡が認められ42日後の平均体重は146.7 g（2/20例）であった。

ONO-1301の3 mg/kgの２回/日、反復経口投与群（2群）はMCT投与後、平均体重は増加して21日後は187.6 g(9/10例）であった。その後、体重が減少し死亡が認められ42日後の平均体重は165.1 g（5/10例）であり、対照群（1群）と比較して有意な差は認められなかった。

ボセンタンの50 mg/kgの２回/日、反復経口投与群（3群）はMCT投与後、体重は増加して21日後の平均体重は192.9 g（9/10例）であった。その後、体重が減少し死亡が認められ42日後の平均体重は167.2 g（3/10例）であり、対照群（1群）と比較して有意な差は認められなかった。

ONO-1301NS剤Aの1 mg/kgの１回/週、間歇静脈内投与群（4群）はMCT投与後、体重は増加して21日後の平均体重は186.7 g（10/10例）であった。その後、体重が減少し死亡が認められ42日後の平均体重は145.4 g(5/10例)であり、対照群と比較して有意な差は認められなかった。

ONO-1301NS剤Bの1 mg/kgの１回/週、間歇静脈内投与群（5群）はMCT投与後、体重は増加して18日後の平均体重は176.5 g（9/10例）であった。その後、体重が減少し死亡が認められ42日後の平均体重は113.3 g（1/10例）であった。重症心不全モデル作製27および30日後において対照群（1群）と比較して有意な体重減少を示した（$p<0.05$）。

ONO-1301の1 mg/kgの１回/週、間歇静脈内投与群（6群）はMCT投与後、体重は増加して21日後の平均体重は190.0 g（10/10例）であった。その後、体重が減少し死亡が認められ42日後の平均体重は192.2 g（2/10例）であり、対照群（1群）と比較して有意な差は認められなかった。

３）総投与量

MCT投与７日後から41日までの35日間に投与された動物あたりの総被験物質量を求めた。

その結果、2群は210 mg/kg/animal（3 mg/kg×2回/日×35日）、3群は3500 mg/kg/animal（50 mg/kg×2回/日×35日）、4群～6群は5 mg/kg/animal（1 mg/kg/週×5回）であった。4群は2群と比較し、5/210（1/42）投与量にて同等の効果を示した。

Ｄ．考察

疾患特異的なDDSナノスフェアー製剤として、新規ONO-1301ナノスフェアー製剤（ONO-1301NS）を作製し、間歇静脈内投与により、より汎用性の高い疾患特異的（DDS）な重症心不全治療剤開発を目的とした治療法の開発を検討した。

２種の特性の異なるONO-1301NS製剤を作製し、間歇静脈内投与によりMCT誘発重症心不全モデルでの生存率を比較検討することにより、各種製剤のDDS効果を確認した。

陽性対照物質として用いたET-1拮抗剤であるボセンタンは肺高血圧治療剤として臨床的に使用されており、MCT誘発心不全モデルにおいて有効性を確認しているが、これらはMCT投与直後からの反復経口投与である。今回、MCT投与７日後からの治療投与においては、有意な延命効果が確認出来なかった。一方、ONO-1301の3 mg/kg（2群）の1日2回反復投与での最終生存率は50％であり、対照群（1群）と比較して有意な延命効果が確認された。また、ONO-1301NS剤Aの1 mg/kg（4群）も週1回の間歇静脈内投与においても、最終生存率は50％であり、ONO-1301と同等の延命効果を示した。

ONO-1301NS剤（A）の1 mg/kg/週間歇静脈内投与（４群）はONO-1301の3 mg/kgの２回/日、反復経口投与（2群）と比較し、総投与量として1/42投与量において、同等な延命効果を示すことにより、NS製剤でのDDS効果が確認された。

一方、ONO-1301原薬（６群）、及びONO-1301NS剤（B）の1 mg/kg/週間歇静脈内投与群（５群）は効果を示さなかった（Table１）。

以上の結果より、重症心不全モデルに対して心不全発症後（MCT投与７日後）からの投与においてONO-1301反復経口投与およびONO-1301NS剤A間歇静脈内投与は治療的な投与により有意な延命効果を示し、重症心不全（肺高血圧症）に対する治療効果を有する可能性が示唆された。

Ｅ．結論

重症心不全モデル対して、ONO-1301NS製剤（A）の週１回間歇静脈内投与は、ONO-1301の１日２回反復経口投与と同等の治療効果を示した。ONO-1301の総投与量はNS剤にすることにより1／42投与量で同等の効果を示すことから、ONO-1301NS製剤の間歇静脈内投与によりONO-1301が疾患局所に集積（DDS）されるため、少量投与により有効性を発揮する可能性が示唆された。

ONO-1301NS製剤の間歇静脈内投与は、疾患局所特異的（DDS）であり、安全性、汎用性、経済性、利便性に優れた重症心不全治療剤に成り得ることが示唆された。

Ｆ．健康危険情報

該当なし

Ｇ．研究発表

１．論文発表

1）「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.」
Shirasaka T, Miyagawa S, Fukushima S, Saito A, Shiozaki M, Kawaguchi N, Matsuura N, Nakatani S, Sakai Y, Daimon T, Okita Y, Sawa Y.
J Thorac Cardiovasc Surg. 2013 Aug;146(2):413-21.
2）「Sustained-release delivery of prostacyclin analogue enhances bone marrow-cell recruitment and yields functional benefits for acute myocardial infarction in mice.」
Imanishi Y, Miyagawa S, Fukushima S, Ishimaru K, Sougawa N, Saito A, Sakai Y, Sawa Y.
PLoS One. 2013 Jul 19;8(7):1-8
3）「Synthetic prostacyclin agonist, ONO1301, enhances endogenous myocardial repair in a hamster model of dilated cardiomyopathy: a promising regenerative therapy for the failing heart.」
Ishimaru K, Miyagawa S, Fukushima S, Saito A, Sakai Y, Ueno T, Sawa Y.
J Thorac Cardiovasc Surg. 2013 Dec;146(6):1516-25.
4）「Impact of cardiac support device combined with slow-release prostacyclin agonist in a canine ischemic cardiomyopathy model.」
Kubota Y, Miyagawa S, Fukushima S, Saito A, Watabe H, Daimon T, Sakai Y, Akita T, Sawa Y.
J Thorac Cardiovasc Surg. 2014 Mar;147(3):1081-7.

２．学会発表

1）「A Novel Therapeutic Technology of Long-Acting Prostacyclin Agonist for Mature Porcine Ischemic Heart Model」
Hiroki Mizoguchi ; Shigeru Miyagawa ; Satsuki Fukushima ; Atsuhiro Saito ; Yoshiki Sakai ; Yukiko Imanishi ; Akima Harada ; Takayoshi Ueno ; Koich Toda ; Toru Kuratani ; Yoshiki Sawa
AHA2013 (American Heart Association)
2013.11.16-20 Dallas,TX,USA

2）「長時間作用型 Prostacyclin agonist の心臓局所投与法の開発・効果に関する実験的検討」
溝口裕規、宮川　繁、福嶌　五月、齋藤　充弘、酒井　芳紀、今西悠基子、原田希摩、西宏之、吉川泰司、上野高義、戸田　宏一、澤　芳樹
第４４回日本心臓血管外科学会　熊本（2014.2.19-21）

H．知的財産権の出願・登録状況(予定を含む)

１．特許取得

該当なし

２．実用新案登録

該当なし

３．その他（今後の予定）

1）「新規ナノスフェアー製剤」

・出願人：大阪大学　等

別紙4

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shirasaka T, Miyagawa S, Fukushima S, Saito A, Shiozaki M, Kawaguchi N, Matsuura N, Nakatani S, Sakai Y, Daimon T, Okita Y, Sawa Y.	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.	J Thorac Cardiovasc Surg	Aug;146(2)	413-21	2013
Imanishi Y, Miyagawa S, Fukushima S, Ishimaru K, Sougawa N, Saito A, Sakai Y, Sawa Y.	Sustained-release delivery of prostacyclin analogue enhances bone marrow-cell recruitment and yields functional benefits for acute myocardial infarction in mice.	PLoS One.	Jul 19;8(7)	1-8	2013
Ishimaru K, Miyagawa S, Fukushima S, Saito A, Sakai Y, Ueno T, Sawa Y.	Synthetic prostacyclin agonist, ONO1301, enhances endogenous myocardial repair in a hamster model of dilated cardiomyopathy: a promising regenerative therapy for the failing heart.	J Thorac Cardiovasc Surg	Dec;146(6)	1516-25	2013
Kubota Y, Miyagawa S, Fukushima S, Saito A, Watabe H, Daimon T, Sakai Y, Akita T, Sawa Y.	Impact of cardiac support device combined with slow-release prostacyclin agonist in a canine ischemic cardiomyopathy model.	J Thorac Cardiovasc Surg.	Mar;147(3)	1081-7	2014

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

Tomonori Shirasaka, MD,[a] Shigeru Miyagawa, MD, PhD,[b] Satsuki Fukushima, MD, PhD,[b] Atsuhiro Saito, PhD,[b] Motoko Shiozaki, PhD,[b] Naomasa Kawaguchi, PhD,[c] Nariaki Matsuura, MD, PhD,[c] Satoshi Nakatani, MD, PhD,[d] Yoshiki Sakai, PhD,[e] Takashi Daimon, PhD,[f] Yutaka Okita, MD, PhD,[a] and Yoshiki Sawa, MD, PhD[b]

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,

From the Division of Cardiovascular Surgery,[a] Kobe University Graduate School of Medicine, Kobe; the Division of Cardiovascular Surgery,[b] Osaka University Graduate School of Medicine, Suita; the Department of Molecular Pathology,[c] Graduate School of Medicine and Health Sciences, Division of Functional Diagnostics,[d] Department of Health Sciences, Osaka University Graduate School of Medicine, Osaka; Ono Pharmaceutical Company Ltd,[e] Osaka; and the Division of Biostatistics,[f] Hyogo College of Medicine, Hyogo, Japan.

Disclosures: Yoshiki Sakai is an employee of ONO pharmaceutical Co Ltd. All other authors have nothing to disclose with regard to commercial support.

Received for publication July 27, 2012; revisions received Sept 8, 2012; accepted for publication Oct 2, 2012; available ahead of print April 1, 2013.

Address for reprints: Yoshiki Sawa, MD, PhD, Osaka University Graduate School of Medicine, Suita, Japan, E1: 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan (E-mail: sawa@surg1.med.osaka-u.ac.jp).

0022-5223/$36.00

Copyright © 2013 by The American Association for Thoracic Surgery

http://dx.doi.org/10.1016/j.jtcvs.2012.10.003

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 pentobarbital (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 pentobarbital (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 $\pm$ 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% $\pm$ 1.7 % vs 19% $\pm$ 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 $\pm$ 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 $\pm$ 2.4 vs 31 $\pm$ 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 $\pm$ 2.3 vs 34 $\pm$ 1.4; $P < .05$; Figure 2, *A*). E/E′ after ONO1301SR injection was significantly smaller than that after PLGA injection (E/E′, 11 $\pm$ 1.2 vs 16 $\pm$ 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 $\pm$ 0.9 vs 55 $\pm$ 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

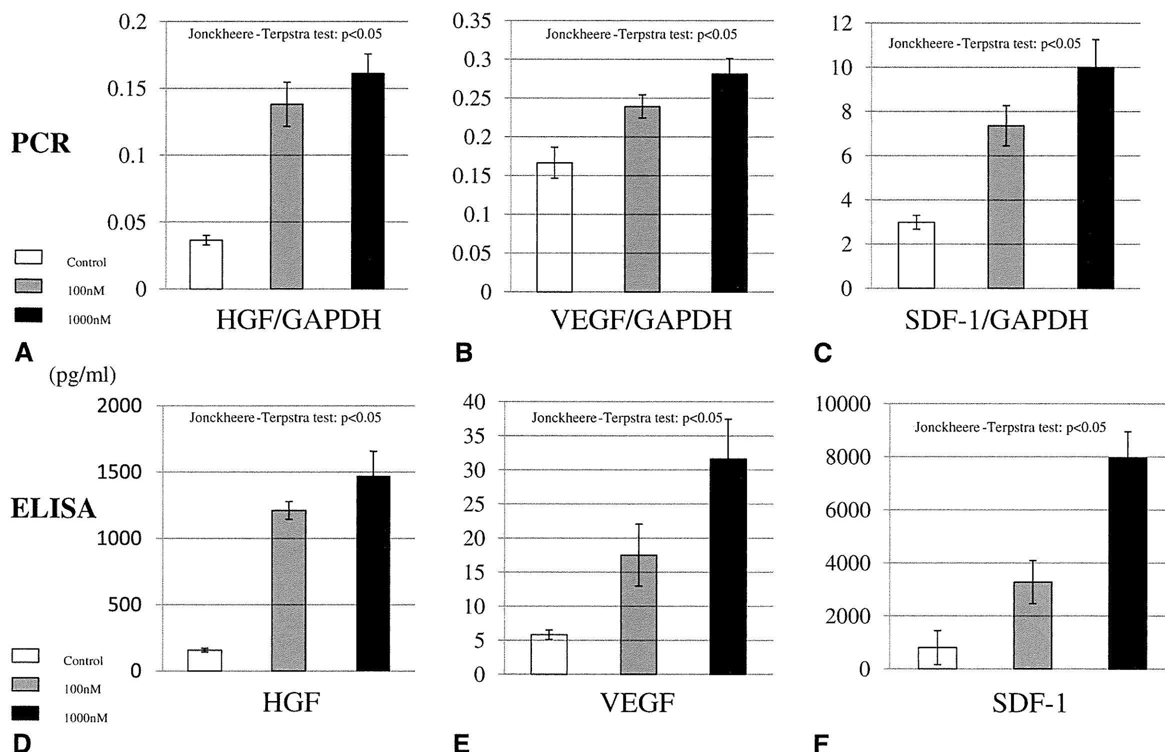


FIGURE 1. PCR and ELISA analysis in vitro showed that messenger RNA levels for HGF (A and D), VEGF (B and E), and SDF-1 (C and F) increased in a dose-dependent manner in NHDF cultured with ONO1301. *PCR*, Polymerase chain reaction; *ELISA*, enzyme-linked immunosorbent assay; *NHDF*, normal human dermal 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.

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

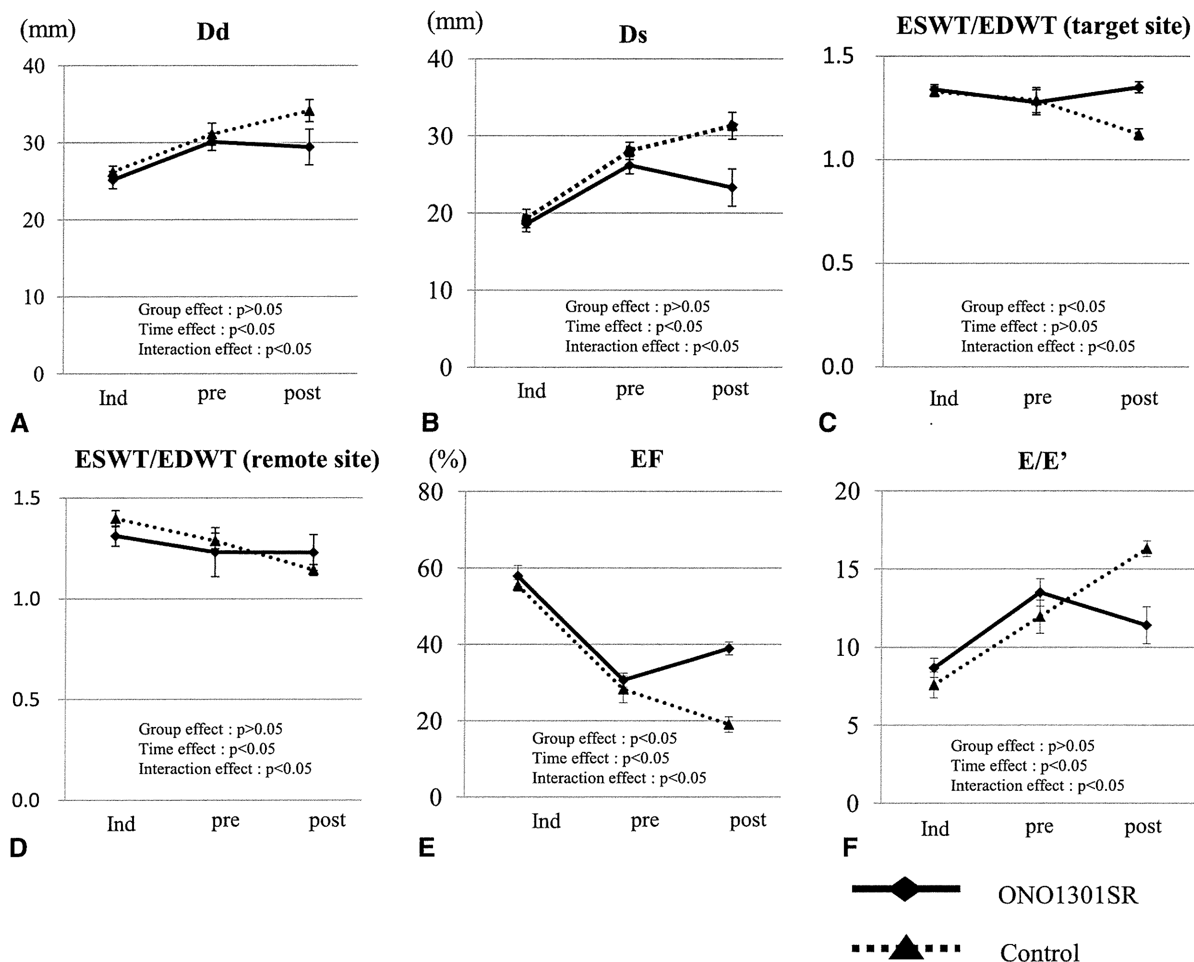


FIGURE 2. Echocardiography (A-F) showed ONO1301SR significantly improved distressed cardiac function. Note that ESWT/EDWT, reflecting on the radial strain of myocardium, was significantly more recovered in the ONO1301SR group than in the control group and that the clinical impact was more prominent in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

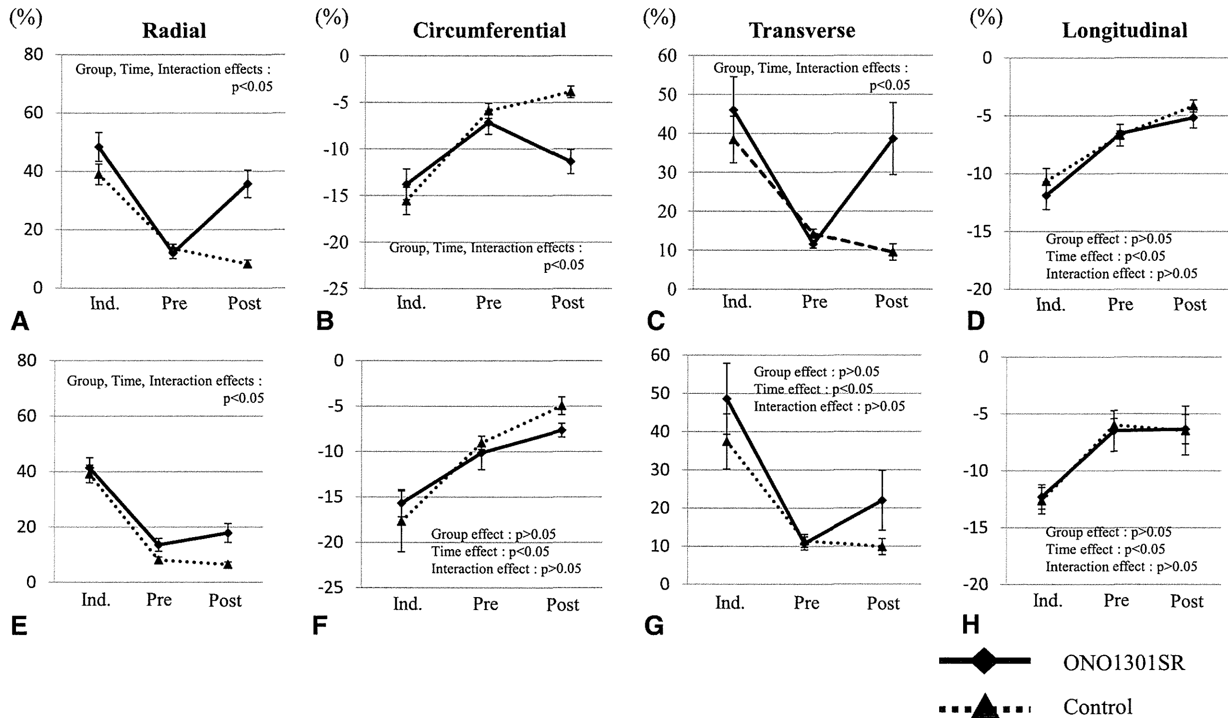


FIGURE 3. Speckle-tracking echocardiography showed that the absolute values of peak radial, circumferential, and transverse strains at the target site in the ONO1301SR group were significantly higher than in the control group (A-D) while all of them but radial strains at the remote site in the ONO1301SR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

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,

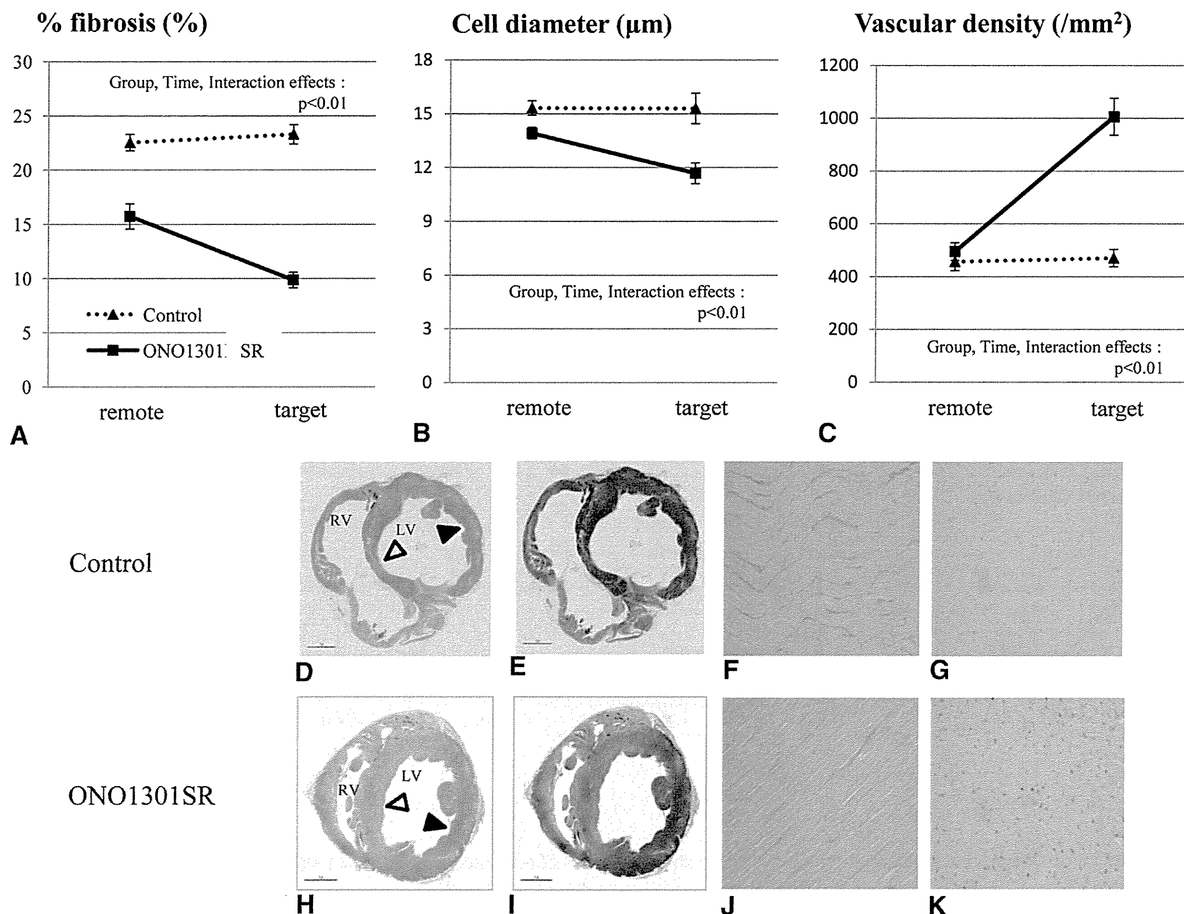


FIGURE 4. Histopathology; quantitative evaluation of interstitial fibrosis (A), mean cell diameter (B), vascular density (C), and representative micrograph of the Control group (D-G) and the ONO1301SR group (H-K). D and H, Hematoxylin and eosin staining. E and I, Masson trichrome staining. F and J, Sirius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

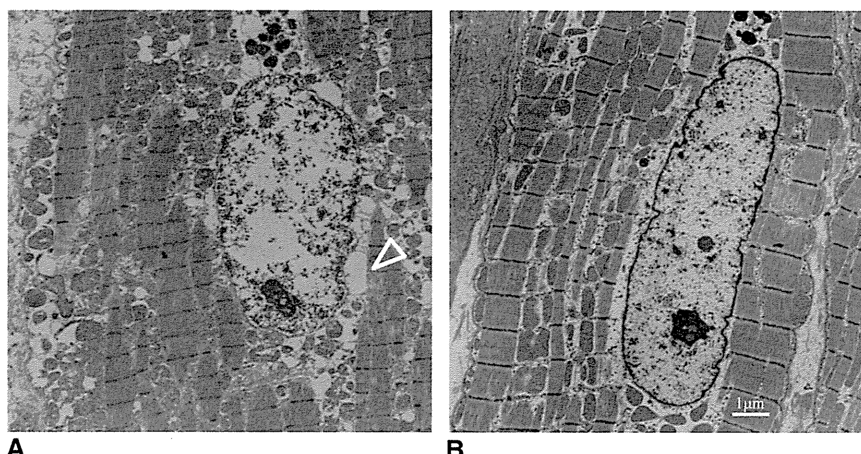


FIGURE 5. Electron microscopy revealed that the myocardium of the control group (A) showed prominent swelling or disruption of mitochondria, intracellular or perinuclear edema, and sarcoplasmic vacuoles resulting from dilation of sarcoplasmic reticulum *(arrowhead)*. On the other hand, mitochondria in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

We thank Masako Yokoyama, Yuka Fujiwara, and Shigeru Matsumi for their excellent technical assistance.

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.

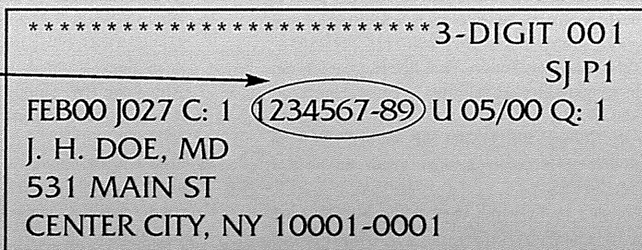
21. Iwata H, Nakamura K, Sumi M, Ninomiya M, Sakai Y, Hirata Y, et al. Local delivery of synthetic prostacycline agonist augments collateral growth and improves cardiac function in a swine chronic cardiac ischemia model. *Life Sci.* 2009;85:255-61.
22. Effect of stromal-cell-derived factor 1 on stem-cell 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.

Access to ***The Journal of Thoracic and Cardiovascular Surgery Online*** is reserved for print subscribers!

Full-text access to ***The Journal of Thoracic and Cardiovascular Surgery Online*** is available for all print subscribers. To activate your individual online subscription, please visit ***The Journal of Thoracic and Cardiovascular Surgery Online,*** point your browser to ***http://www.mosby.com/jtcvs,*** follow the prompts to **activate your online access,** and follow the instructions. To activate your account, you will need your subscriber account number, which you can find on your mailing label (*note:* the number of digits in your subscriber account number varies from 6 to 10). See the example below in which the subscriber account number has been circled:

Sample mailing label

This is your subscription account number



Personal subscriptions to ***The Journal of Thoracic and Cardiovascular Surgery Online*** are for individual use only and may not be transferred. Use of ***The Journal of Thoracic and Cardiovascular Surgery Online*** is subject to agreement to the terms and conditions as indicated online.

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

Yukiko Imanishi[1], Shigeru Miyagawa[1], Satsuki Fukushima[1], Kazuhiko Ishimaru[1], Nagako Sougawa[1], Atsuhiro Saito[2], Yoshiki Sakai[3], Yoshiki Sawa[1]*

1 Department of Cardiovascular Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan, **2** Medical Center for Translational Research, Osaka University Hospital, Osaka, Japan, **3** Research Headquarters, ONO Pharmaceutical CO., LTD., Osaka, Japan

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 $23\pm1\%$; $n=22$) compared to the vehicle group ($19\pm1\%$; $n=20$; $P=0.004$). 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.

Citation: Imanishi Y, Miyagawa S, Fukushima S, Ishimaru K, Sougawa N, et al. (2013) Sustained-Release Delivery of Prostacyclin Analogue Enhances Bone Marrow-Cell Recruitment and Yields Functional Benefits for Acute Myocardial Infarction in Mice. PLoS ONE 8(7): e69302. doi:10.1371/journal.pone.0069302

Editor: Toru Hosoda, Tokai University, Japan

Received February 8, 2013; **Accepted** June 6, 2013; **Published** July 19, 2013

Copyright: © 2013 Imanishi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was funded by grant-in-aid for Core-to-Core Program (21003) from the Japan Society for the Promotion of Science (http://jsps-osaka-u.jpn.org/en/index.html), early-stage and exploratory clinical trial centers project from the Ministry of Health (http://jsps-osaka-u.jpn.org/en/index.html), Labour and Welfare, Health and Labour Sciences Research Grant (H23-002, http://jsps-osaka-u.jpn.org/en/index.html), and from New Energy and Industrial Technology Development Organization (P10004, http://www.nedo.go.jp/english/index.html). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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.

* E-mail: sawa@surg1.med.osaka-u.ac.jp

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$\times$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

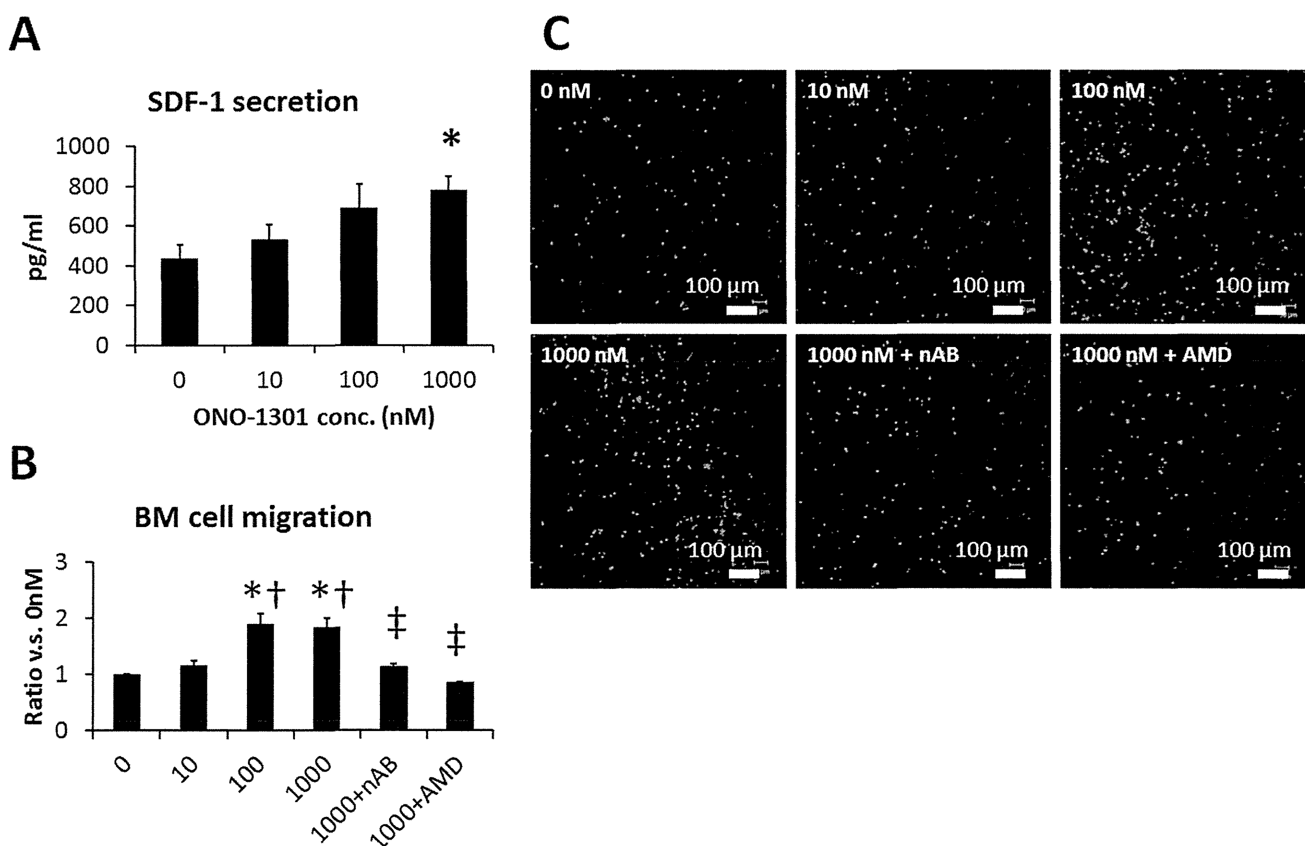


Figure 1. ONO-1301 enhanced SDF-1 secretion and BMC migration via SDF-1/CXCR4 signaling *in vitro*. NHDFs were stimulated with ONO-1301 for 72 hours, then the SDF-1 concentration in the culture medium was determined by ELISA (n = 3 each, *$P<0.05$ vs. 0 nM). A) Number of BMCs that migrated toward the 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.
doi:10.1371/journal.pone.0069302.g001

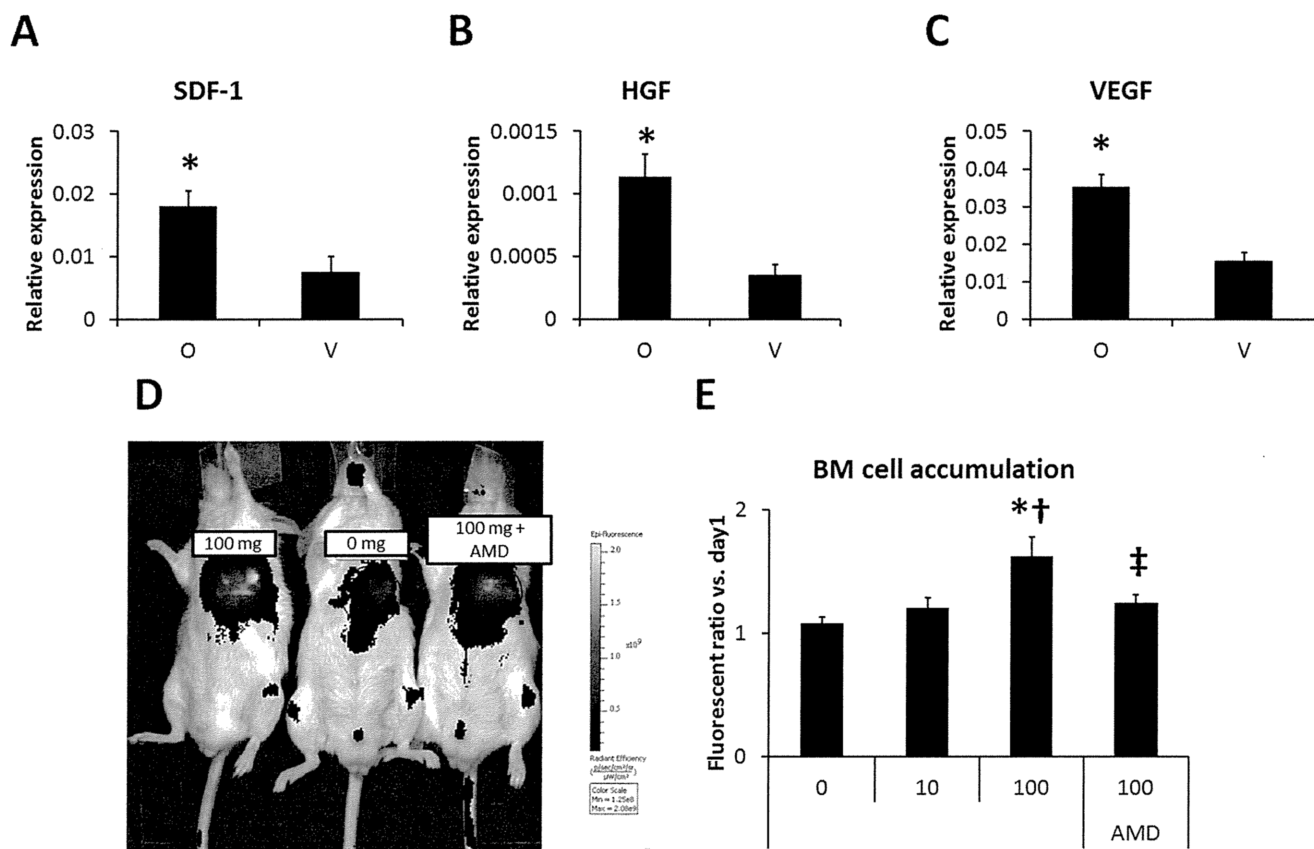


Figure 2. ONO-1301 enhanced SDF-1 secretion and BMC migration via SDF-1/CXCR4 signaling after MI. A–C) The SDF-1, HGF, and VEGF expression at the border zone of the infarcted area was measured by quantitative RT-PCR. The expression levels of these cytokines were higher in the ONO-1301-treated (O) group compared to the vehicle (V) group. (O group, n = 7; V group, n = 7–8; *$P<0.05$ vs. V group). The expression relative to GAPDH is shown. D) BMC migration to ONO-1301-treated infarcted myocardium was evaluated using IVIS. Representative picture of IVIS at day 3. Left: 100 mg/Kg, Center: 0 mg/Kg, Right: 100 mg/Kg+AMD3100 (AMD). E) The number of accumulated BMCs was greater in the 100 mg/kg ONO-1301-treated infarcted heart compared to the 0 and 10 mg/kg ONO-1301-treated infarcted heart. When BMCs treated with AMD were injected, the BMC accumulation decreased in the 100 mg/Kg ONO-1301-treated infarcted heart compared with the untreated-BMC-injected heart (0 mg/Kg, n = 4; 10 mg/Kg, n = 8; 100 mg/Kg, n = 5; 100 mg/Kg+AMD3100, n = 4; *$P<0.05$ vs. 0 mg/Kg, †$P<0.05$ vs. 10 mg/Kg, ‡$P<0.05$ vs. 100 mg/Kg).
doi:10.1371/journal.pone.0069302.g002

of GFP with either of these markers was observed (figure S3 in File S1).

Therapeutic Effects of ONO-1301 Administration on Cardiac Performance, Survival, and LV-remodeling at 4 Weeks Post-MI

ONO-1301 was detected in the plasma of blood samples from the ONO-1301-treated group 3 weeks after treatment (figure S4 in File S1). The cardiac functions in the MI mice with and without following ONO-1301 treatment were evaluated. Mortality was substantial until 14 days post-LAD ligation in the vehicle group, and similar mortality levels were observed with non-treated MI mice [11]. In contrast, in the ONO-1301-treated group, there was little mortality 7 days after MI, and thus a difference in survival (Fig. 4A). Cardiac performance was evaluated by 2D echocardiography 4 weeks after implantation. The LVEDA was smaller in the ONO-1301-treated group than in the vehicle group, but the difference was not significant. In contrast, the LVESA was significantly smaller, and the LVFAC was significantly greater, in the ONO-1301-treated group than in the vehicle group (Fig. 4B). In the histological analysis, the vehicle group showed a typical MI with a large anterior LV scar and dilatation of the LV cavity. By comparison, the LV of the ONO-1301-treated group was less dilated, and the anterior wall was thicker (Fig. 4C, D). The infarcted area and percent fibrosis were significantly smaller in the ONO-1301-treated than in the vehicle-treated group (Fig. 4C, E–G).

Discussion

Here, we showed that ONO-1301 promotes BMC accumulation in the injured myocardium. *In vitro*, ONO-1301 enhanced SDF-1 expression, and BMC migration was greater to conditioned medium obtained from ONO-1301-stimulated cells. The enhanced migration was diminished by blocking SDF-1/CXCR4 signaling. Consistent with the *in vitro* experiments, ONO-1301 enhanced the SDF-1 expression of myocardial tissue. High ONO-1301 accelerated the BMC accumulation after MI in a SDF-1/CXCR4-dependent manner. Some BMCs in the infarcted myocardium differentiated into capillary structures within 7 days. Furthermore, the sustained-release delivery of ONO-1301 in the infarcted myocardium also led to functional improvements following MI. Our data suggest that ONO-1301 is a novel inducer of BMC recruitment, and that ONO-1301 treatment may be a promising therapeutic strategy for the clinical treatment of MI.

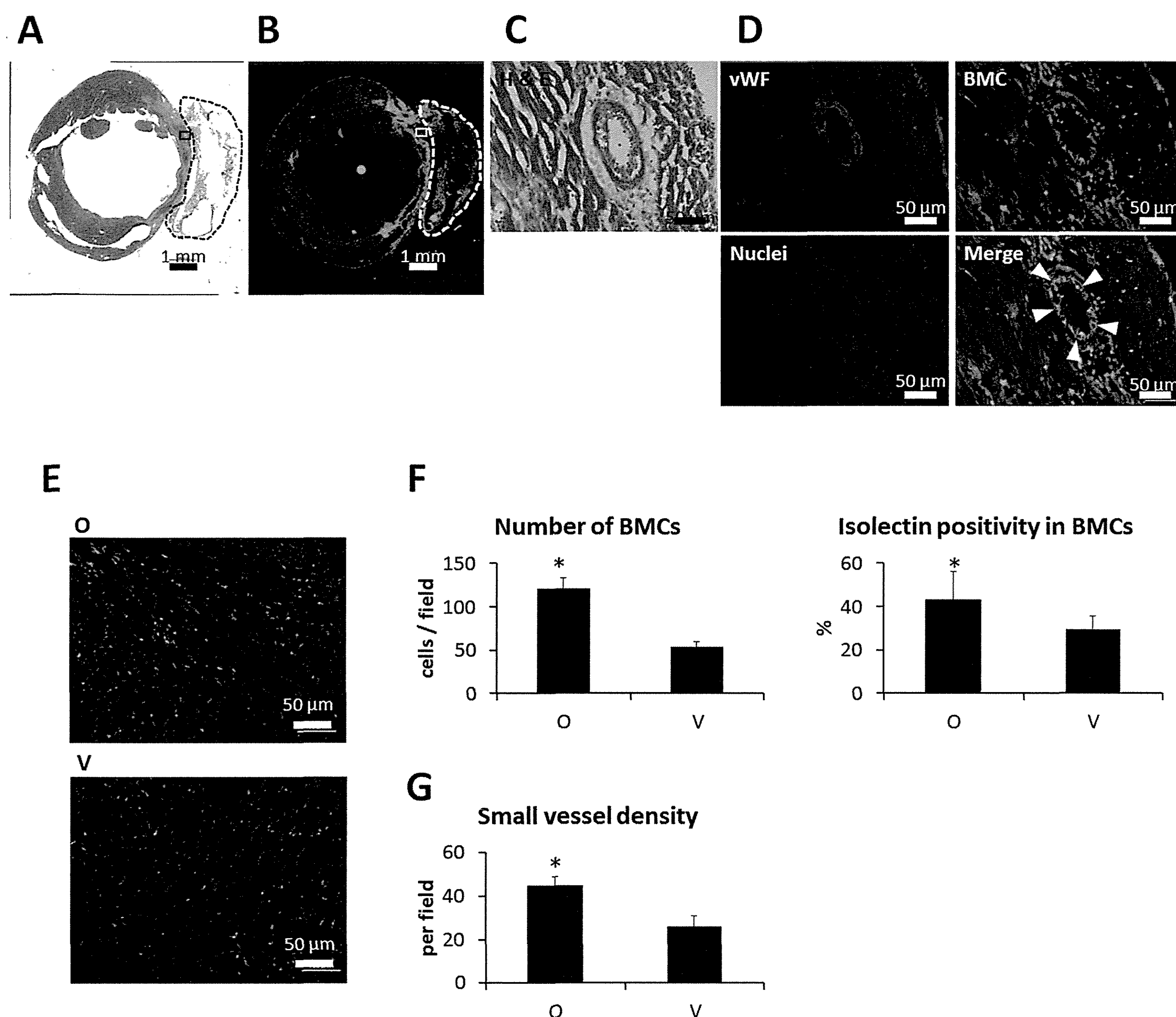


Figure 3. BMCs differentiated into capillary structures in the infarcted area after MI and ONO-1301 treatment. Representative macro image of H and E staining seven days after MI and ONO-1301 treatment. The transplanted sheet is enclosed by a dashed line. A) Serial section of A. The BMCs displayed GFP. B) High-magnification image of the boxed region in A. C) Serial section of C. Arrowheads indicate vWF-expressing BMCs. Red indicates vWF; green, BMCs; and blue, nuclei. D) Representative images of isolectin-stained BMCs seven days after MI and ONO-1301 treatment. E) BMC accumulation and percentages of isolectin-positive BMCs. The number of BMCs that accumulated in the infarcted myocardium was greater in the ONO-1301-treated (O) group than in the vehicle (V) group. The percentage of isolectin-positive BMCs was also greater in the O group than in the V group. *$P<0.05$ vs. V group. F) Small vessel density. Small vessels were detected by CD31 immunostaining. The density of small vessels in the O group was greater than in the V group. *$P<0.05$ vs. V group.
doi:10.1371/journal.pone.0069302.g003

It is difficult to understand the whole mechanism underlying the functional improvements induced by ONO-1301. It was already reported that ONO-1301 enhances the expression of angiogenic factors HGF and VEGF, leading to angiogenesis and the suppression of fibrosis progression [7,8,9]. In this study, we discovered an alternative mechanism for ONO-1301's therapeutic efficacy in the acute MI mouse, in which the upregulation of SDF-1 promotes BMC accumulation. Stem-cell recruitment and homing are regulated by the interplay of cytokines, chemokines, and proteases. In particular, the SDF-1/CXCR4 axis is central for the mobilization of stem cells from the bone marrow and their homing to ischemic tissues [12]. In the case of ischemic insult, SDF-1 is released by the injured tissue and stimulates the mobilization of progenitor cells from the bone marrow [1,13]. Furthermore, prostaglandins have been reported to facilitate BMC mobilization via upregulation of CXCR4 expression [14,15]. In our experimental setting, ONO-1301 was detected from peripheral blood samples 3 weeks after treatment (Fig. S4 in File S1), suggesting that ONO-1301 may similarly act on the bone marrow to promote the BMC mobilization. Thus, BMC recruitment in the injured myocardium may be enhanced by the upregulation of SDF-1 in cardiac fibroblasts and by the direct upregulation of CXCR4 in BMCs located in the bone marrow. In addition, recent reports show the possibility of endogenous regeneration in the injured heart, including proliferation of postnatal cardiomyocytes and cardiac stem cells [16,17,18,19]. While we were unable to

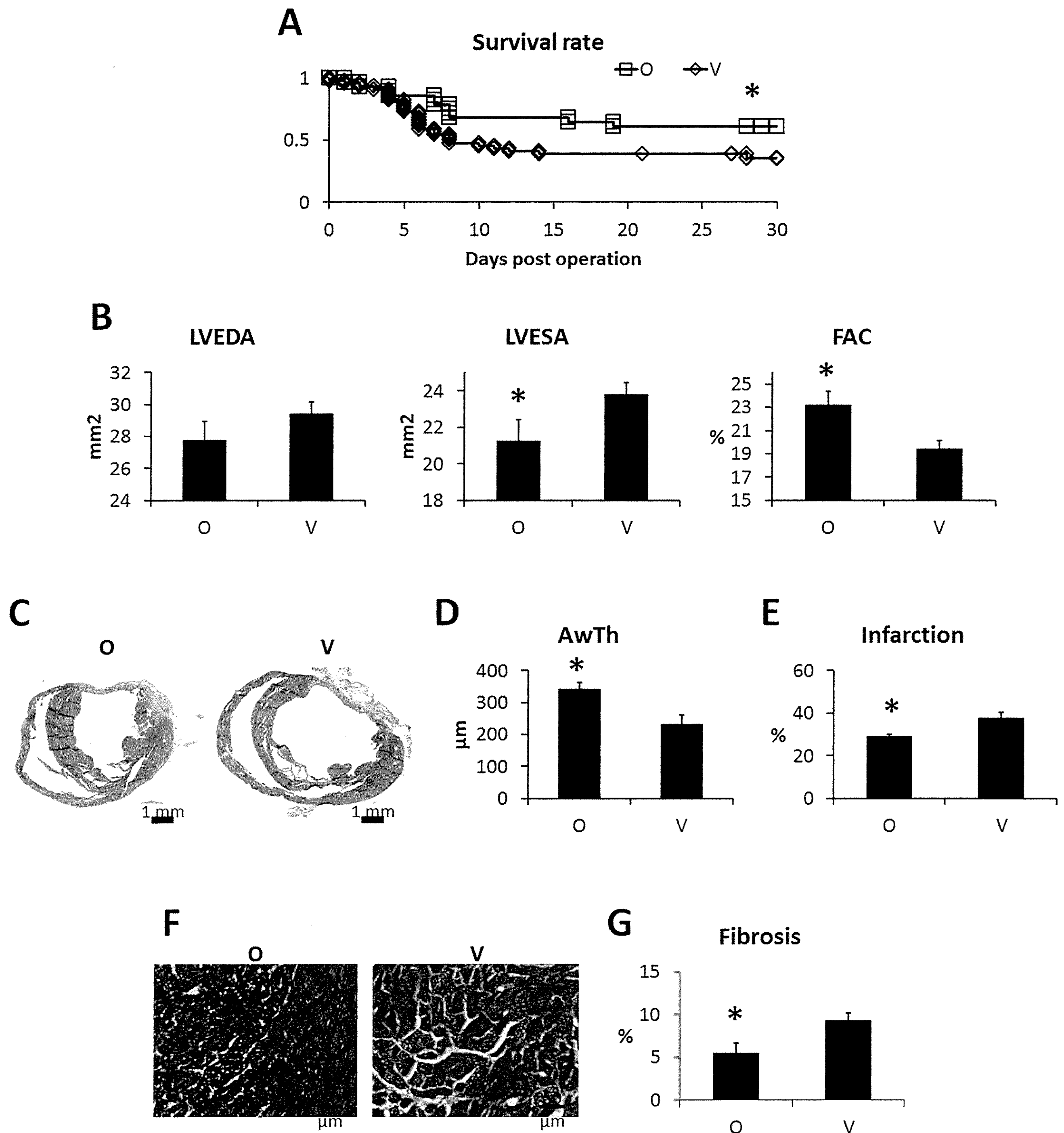


Figure 4. ONO-1301 treatment improved the cardiac performance and survival rate after MI. Survival rates after treatment. The ONO-1301-treated (O) group (n = 33) showed significantly better survival than the vehicle (V) group (n = 48). *$P<0.05$ vs. V group. A) Evaluation of cardiac performance 4 weeks after treatment. In the O group, the LVESA was smaller, and the FAC was significantly higher compared to the V group (O group, n = 22; V group, n = 20; *$P<0.05$ vs. V group). B) Representative macro images from each group. C) Quantification of anterior wall thickness. Anterior wall thickness was significantly thicker in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group. D) Quantification of percent infarction. Infarction was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group. E) Representative Masson trichrome staining images at the border zone. F) Quantification of fibrosis. Fibrosis at the border zone was significantly smaller in the O group (n = 6) compared to the V group (n = 4). *$P<0.05$ vs. V group.
doi:10.1371/journal.pone.0069302.g004

detect newly-generated cardiomyocytes derived from BMCs in this study, it would be interesting to evaluate the possibility of cardiomyogenesis involving other cell types.

We observed massive BMC accumulation 7 days after MI, including in the infarcted ventricular wall, where they provided structural support in place of the necrotic cardiomyocytes. The