厚生科学研究費補助金（ヒトゲノム・再生医療等研究事業）

分担研究報告書

組織幹の動態とその制御因子に関する研究

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研究要旨　血中前駆細胞は強い傷害後の血管修復と病態形成に関与することが再確認された。骨髄中の間葉系もしくはより未分化幹細胞から前駆細胞は派生しており、造血幹細胞からの形質転換分化の可能性は少ないと思われる。「骨髄由来血管前駆細胞」が血管病予防のための新たな標的になることが明らかになった。

A．研究目的

閉塞性血管病は局所の細胞の分化、増殖によって生じると考えられている。私たちは、移植後動脈硬化、血管形成術後再狭窄、高脂血症による動脈硬化のモデルを用いて、血液中に動員された骨髄由来前駆細胞が傷害後の血管に定着し、内皮細胞もしくは平滑筋細胞に分化して病変形成に関与することを報告した。この説は世界で広く受け入れられるようになったが、一方では骨髄由来細胞の可塑性に疑問を投げかける報告も多くみられている。特に骨髄中の造血幹細胞の可塑性に関しては非常に激しい論争がなされている。血液中の骨髄由来前駆細胞が血管病変形成に関与するかどうか、いくつかの方法を用いて再検証してみた。

B．研究方法

(1) 異なる血管傷害モデルを用いての検討

野生型マウスに致死量のX線(9Gy)を照射し、GFPマウスもしくはLacZマウスの骨髄を移植した。一匹の骨髄移植マウスの三つの異なる血管に、同時に別々の異なる傷害を加えた。ワイヤー傷害(Wire Injury)、頚動脈の結紮（Ligation)、ポリエチレンチューブの大腿動脈周囲への留置（Cuff)の三種類の異なるモデルで手術を行い、４週後に骨髄由来の病変への取りこまれ方を検討した。

(2) 一個の造血幹細胞を用いた骨髄置換マウスの解析

骨髄細胞のうち、血管の修復と病変形成に関与する分画を検討した。野生型マウスの骨髄を、①全骨髄$1x10^6$個(TBM群)、② 造血幹細胞が大部分を占めるc-Kit^+, Sca-1^+, Lin^-分画$1x10^3$個（KSL群）③ 高度に純化した造血幹細胞1個(HSC群)によって置換した。その後、血管にワイヤーを用いた傷害を加え、病変への骨髄細胞の取り込まれ方を比較した。

(3) Parabiosis モデルを用いての検討

従来の骨髄置換法においては、致死量放射線照射を用いたレシピエント骨髄の破壊が必須である。しかし、放射線照射は骨髄以外のさまざまなレシピエント臓器に影響をもたらし、生理的な骨髄細胞の標識方法ではない。また、移植した骨髄細胞が造血系を完全に置換することは認められているが、他の間葉系システムも生理的に再構築しうるかどうかは不明である。そこで、照射を伴わないで骨髄と血液細胞を標識する方法として、二つのマウスを皮下で結合させるというparabiosisのモデルを樹立した。このモデルを用いて、血管傷害と高脂血症性血管傷害モデルを施し、パートナー由来細胞の血管病変への関与を検討した。

（倫理面への配慮）

本研究では既に確立された細胞と実験動物疾患モデルを用いて検討する。動物は換気、給

餌等の完備した施設で飼育し、学内もしくは研究所内の規定に適合する条件で実験を行うため倫理的な問題はない。

C．研究結果

(1) 異なる血管傷害モデルを用いての検討

ワイヤー傷害（Wire Injury)、頚動脈の結紮(Ligation)、ポリエチレンチューブの大腿動脈周囲への留置（Cuff）何れのモデルによってもアクチン陽性細胞からなる新生内膜が形成された。Wire Injury では多くの骨髄細胞が取り込まれていたが、Ligation では非常に少なかった。また、Cuff では周囲の炎症細胞としては骨髄由来細胞が存在したが、新生内膜には殆ど取り込まれていなかった。骨髄由来細胞の取り込まれる程度は、組織損傷の程度とその後のケモカイン、サイトカインの発現量と相関していた。特に wire injury 後の大腿動脈では、内皮はほぼ完全に剥離され中膜の細胞はアポトーシスにより消失していた。このような強い傷害後は、修復に必要な細胞が局所に残存せず遠隔の幹細胞が動員されざるをえなくなると考えられた。

(2) 一個の造血幹細胞を用いた骨髄置換マウスの解析

TBM, KSL, HSC どの群においても末梢の血液細胞は移植細胞由来のものに再構築されていた。TBMもしくはKSLで骨髄を置換したマウスでは、傷害後の血管病変に骨髄由来細胞が数多く認められた。骨髄由来細胞の多くの細胞は、血管平滑筋細胞もしくは内皮細胞のマーカーを発現していた。一方、一個の造血幹細胞を移植したHSC群では、病変には骨髄由来細胞が関与することは殆ど認められなかった。以上より、造血幹細胞より未分化な骨髄細胞もしくは間葉系細胞から、血管前駆細胞が分化している可能性が高いと考えられた。

(3) Parabiosisモデルを用いての検討

皮下の結合によって、従来から知られているように液性因子が交流するばかりでなく、末梢血、骨髄細胞も二マウス間を交流していた。すなわち、GFPマウスと野生型マウスを結合すると、７－１０日には野生型マウスの末梢血の約50%がGFP陽性になっていた。3-4ヶ月後には、骨髄においてもほぼ50%のキメリズムが確認された。片方のマウスの血管にワイヤー傷害を加えると、パートナー由来の細胞が新生内膜形成に関与していた。

D．考察

血液中の前駆細胞が強度傷害後の血管の修復と病変形成に関わる現象が再確認された。前駆細胞としてはいろいろな細胞分画が混在している可能性が高いが、造血幹細胞からの形質転換分化現象は少ないようである。各種病態における前駆細胞の動態とその制御機構を現在研究している。

E．結論

流血中には骨髄由来の血管前駆細胞が存在し血管病の病態生理に関与していると考えられる。その動員、定着、分化、増殖に関する研究は、血管病の新たな治療法開発に貢献すると期待される。

F．健康危険情報

特になし。

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H. 知的財産権の出願・登録状況

1．特許取得　なし

2．実用新案登録　なし

3．その他

研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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研究成果の刊行物・別刷

A synthetic small molecule, ONO-1301, enhances endogenous growth factor expression and augments angiogenesis in the ischaemic heart

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ABSTRACT

It has been shown previously that administration of angiogenic growth factors as genes or proteins can augment collateral growth in ischaemic tissues. In the present study, we have investigated the effect of ONO-1301, a synthetic prostacyclin agonist with thromboxane-synthase-inhibitory activity, on expression of endogenous growth factors and angiogenesis. ONO-1301 induced secretion of HGF (hepatocyte growth factor) and VEGF (vascular endothelial growth factor) from cultured normal human dermal fibroblasts in a dose-dependent manner. Dibutyryl cAMP, an analogue of cAMP, and forskolin, an adenylate cyclase activator, mimicked the effect of ONO-1301. Conversely, Rp-cAMP (adenosine 3′,5′-cyclic monophosphorothioate), an inhibitor of cAMP, partially inhibited the effect of ONO-1301, suggesting that cAMP mediated the effect of ONO-1301 in up-regulating the expression of HGF and VEGF, at least in part. ONO-1301 promoted tube-like formation by HUVECs (human umbilical vein endothelial cells) when co-cultured with fibroblasts, and the angiogenic effect of ONO-1301 was abrogated by administration of a neutralizing antibody against HGF or VEGF. To generate a slow-releasing form of ONO-1301, ONO-1301 was mixed with poly(DL-lactic-co-glycolic acid). The slow-releasing form of ONO-1301 was injected directly into the ischaemic myocardium of mice immediately after ligation of the left anterior descending artery. The slow-releasing form of ONO-1301 up-regulated HGF and VEGF expression and increased capillary density in the border zone (342.7 ± 29.7 capillaries/mm^2 in controls compared with 557.2 ± 26.7 capillaries/mm^2 in treated animals; $P < 0.01$) at 7 days. The slow-releasing form of ONO-1301 ameliorated left ventricular enlargement after 28 days and improved survival rate. In conclusion, our results indicate that ONO-1301 up-regulated endogenous growth factors and promoted angiogenesis in response to acute ischaemia. Therefore ONO-1301 might have a therapeutic potential in treating ischaemic diseases.

Key words: angiogenesis, cardiovascular system, hepatocyte growth factor (HGF), ischaemia, prostacyclin, regenerative medicine, vascular endothelial growth factor (VEGF).

Abbreviations: EBM, endothelial growth medium; FBS, fetal bovine serum; FS, fractional shortening; HASMC, human aortic smooth muscle cell; HGF, hepatocyte growth factor; HUVEC, human umbilical vein endothelial cell; LV, left ventricular; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; MI, myocardial infarction; NHDF, normal human dermal fibroblast; PG, prostaglandin; PLGA, poly(DL-lactic-co-glycolic acid); Rp-cAMP, adenosine 3′,5′-cyclic monophosphorothioate; RT-PCR, reverse transcriptase-PCR; SR-ONO, slow-releasing form of ONO-1301; VEGF, vascular endothelial growth factor.

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INTRODUCTION

Therapeutic angiogenesis has emerged as a promising therapy to treat ischaemic cardiovascular diseases [1]. Administration of growth factors as genes or recombinant proteins has been shown to significantly augment collateral growth in ischaemic tissues [2]. Recent evidence suggests that transplantation of endothelial progenitor cells or total bone marrow cells augments collateral development of ischaemic tissues [3]. As the number of incorporated cells with an endothelial phenotype in ischaemic tissues is generally quite low [4], it was hypothesized that the release of pro-angiogenic factors may mediate the efficacy of autologous cell transplantation [5]. Thus supplementation of angiogenic cytokines may represent a standard strategy in therapeutic angiogenesis. Although it remains to be determined which factor(s) would be the most efficient for clinical use [6], it has been suggested that a combination of several cytokines might be more effective [7].

PGs (prostaglandins) consist of a variety of bioactive substances that play an important role in maintaining local tissue homoeostasis and evoking inflammatory responses. Interestingly, some PGs and their analogues have been shown to stimulate expression of growth factors or cytokines *in vitro* and *in vivo* [8–12]. Matsumoto et al. [8] reported that HGF (hepatocyte growth factor) production by fibroblasts was strongly induced by PGE_1 and prostacyclin, but only slightly by PGE_2 and PGD_2, through transcriptional activation of the HGF gene. It was hypothesized that PGE_1 and prostacyclin, rapidly synthesized after tissue injury, may have a role in tissue repair by inducing HGF expression [8]. In fact, exogenous administration of prostacyclin or its analogues has been shown to protect ischaemic myocardium *in vivo* [13,14]. Indeed, prostacyclin is the major PG released from the ischaemic heart [15]. Prostacyclin analogues have been reported to induce VEGF (vascular endothelial growth factor) expression and enhance angiogenesis *in vivo* in a mouse cornea model [16].

ONO-1301 is a synthetic prostacyclin agonist lacking the typical prostanoid structures, including a five-membered ring and allylic alcohol [17–20]. Prostacyclin and its analogues are not stable *in vivo*, because 15-hydroxyPG dehydrogenase metabolizes their prostanoid structures. In contrast, ONO-1301 is chemically and biologically stable because of the absence of prostanoid structures. Notably, ONO-1301 has thromboxane-synthase-inhibitory activity, because of the presence of a 3-pyridine radical. ONO-1301 exerts long-lasting prostacyclin activity when administered *in vivo* [17–20].

In the present study, we have investigated the effect of ONO-1301 on expression of endogenous growth factors and angiogenesis. ONO-1301 successfully up-regulated the expression of endogenous VEGF and HGF *in vitro* and *in vivo*. Local administration of a slow-releasing form of ONO-1301 augmented angiogenesis and ameliorated LV (left ventricular) enlargement after acute MI (myocardial infarction). Our findings suggest that up-regulation of endogenous growth factors with a small molecule might be useful in therapeutic angiogenesis.

MATERIALS AND METHODS

Animals

Adult male CD1 inbred mice were purchased from SLC Japan [21]. Blood pressure and pulse rate of each mouse were measured with a tail-cuff system (BP-98A; Softron) in conscious animals. In each animal, the mean value of three measurements was used for comparison. All experimental procedures and protocols were approved by the Animal Care and Use Committee of the University of Tokyo and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

ONO-1301 and generation of its slow-releasing form

ONO-1301 was synthesized by ONO Pharmaceutical Co. Ltd as described previously [17–20]. A slow-releasing form of ONO-1301 (SR-ONO) was generated by polymerizing ONO-1301 with PLGA [poly(DL-lactic-co-glycolic acid)]. Briefly, ONO-1301 (5 mg) was mixed with 100 mg of PLGA (M_r, 20000; Wako) in 0.1 % polyvinyl alcohol (1:1 molar ratio of PLGA/glycolic acid). Pellets were washed in distilled water several times and freeze-dried. Particle size of SR-ONO was approx. 25–30 μm in diameter, as determined by a Coulter counter. The releasing rate of ONO-1301 PLGA in 0.2 % Tween 80/sodium phosphate buffer was determined by measuring residual ONO-1301 in the pellets.

Measurement of growth factors produced from cultured human fibroblasts

NHDFs (normal human dermal fibroblasts), purchased from Kurabo, were cultured on 48-well plates in DMEM (Dulbecco's modified Eagle's medium; Sigma) containing 10 % (v/v) FBS (fetal bovine serum). Cells were serum-starved for 24 h and were cultured in the absence or presence of ONO-1301 (0.01–1 μmol/l) for 72 h. Dibutyryl cAMP (Sigma), forskolin (Sigma) or Rp-cAMP (adenosine 3′,5′-cyclic monophosphorothioate; Calbiochem) was added to the medium at the concentrations indicated ($n = 3$ for each group). HUVECs (human umbilical vein endothelial cells), purchased from Cambrex, were cultured on 48-well plates in EBM (endothelial growth medium; Cambrex) containing 2 % (v/v) FBS and endothelial cell growth supplements. HUVECs were serum-starved for 24 h and were cultured in EBM with 0.2 % (v/v) FBS in the absence or presence of ONO-1301 (1 μmol/l) for 72 h ($n = 3$ for each group). HASMCs (human aortic smooth muscle cells), purchased

from Kurabo, were cultured on 48-well plates in human smooth muscle cell growth medium (HuMedia-SG2; Kurabo) containing 5% (v/v) FBS and smooth muscle cell growth supplements. HASMCs were serum-starved for 24 h and were cultured in HuMedia-SG2 with 0.5% (v/v) FBS in the absence or presence of ONO-1301 (1 μmol/l) for 72 h ($n=3$ for each group). Supernatants were harvested to measure concentrations of VEGF and HGF by ELISA (Quantikine; R&D Systems). Total RNA was extracted at 6 h after stimulation. Cells from passages 4–7 were used throughout the study.

Measurement of angiogenic activity *in vitro*

An *in vitro* angiogenesis kit (KZ-1000; Kurabo), consisting of a two-dimensional co-culture system of HUVECs/NHDFs on a 24-well plate, was used according to the manufacturer's instructions [22]. Cells were cultured in the absence or presence of ONO-1301 (0.1 μmol/l), a neutralizing anti-(human HGF) antibody (10 μg/ml; generously given by Dr George F. Vande Woude, Laboratory of Molecular Oncology, Van Andel Institute, Grand Rapids, MI, U.S.A.) [23] and a neutralizing monoclonal anti-(human VEGF) antibody (5 μg/ml; mouse IgG2B, clone 26503; R&D Systems), which recognizes human $VEGF_{165}$ and $VEGF_{121}$. Human recombinant $VEGF_{165}$ (KZ-1300; Kurabo) and human recombinant HGF (294-HG; Kurabo) were used as positive controls in the tube-formation assays. Suramin (Kurabo), an antitrypanosomal agent, was used as an inhibitor of angiogenesis [24]. At 11 days, cells were fixed with 10% formalin and stained using an anti-CD31 antibody. The length of CD31-positive endothelial cells of tube-like structures was quantified in five random high-power fields (×40 magnification) in a blinded fashion using an image analysing system (Kurabo). All assays were performed in triplicate.

Injection of SR-ONO into ischaemic myocardium

MI was induced in 8–10-week-old male CD-1 mice, weighing 35–40 g, by ligating the left anterior descending coronary artery, as described previously [25]. Briefly, mice were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg of body weight). A 24-gauge polyethylene tube was inserted into the trachea and mechanical ventilation was provided by a rodent ventilator (SAR-830; CWE). The chest was opened and the heart was exposed. The left descending coronary artery was ligated by 7-0 nylon suture. Immediately after ligation, 2 mg of SR-ONO ($n=31$), suspended in 50 μl of normal saline with 0.2% Tween 80, was injected directly into the anterior and lateral myocardium around the ischaemic border zone. A total of 2 mg of PLGA microspheres containing no ONO-1301 ($n=29$) was injected as a control. During the study period of 4 weeks, cages were inspected daily for survival analysis after MI. A neutralizing polyclonal goat anti-(mouse VEGF) antibody (0.0025 μg/kg of body weight; R&D system), which recognizes mouse $VEGF_{164}$ and $VEGF_{120}$, or normal goat IgG (0.025 μg/kg of body weight) was administered intraperitoneally at 0, 3, 5 and 7 days.

Echocardiography

Echocardiographic studies were performed under anaesthesia with pentobarbital (50 mg/kg of body weight) before surgery and at 1 and 28 days after MI [26]. An echocardiography system (EnVisor M2540A; Philips Medical System) was used with a dynamically focused 15 MHz linear-array transducer with a depth setting of 1.5 cm. The resolution provided by 15 MHz linear-array transducers has been reported to be high enough to measure LV diameter in a mouse heart beating at a high rate [27]. Two-dimensional images and M-mode tracings were recorded from the short-axis view at the high papillary muscle level. Care was taken not to apply too much pressure to the chest wall. Measurements were done for at least ten beats in a blinded fashion and repeated twice. LVEDD (LV end-diastolic diameter) and LVESD (LV end-systolic diameter) were measured. FS (fractional shortening) was calculated using the following equation: $FS\,(\%) = (LVEDD - LVESD)/LVEDD \times 100$.

Histological analysis

For histological analysis, mice were killed by intraperitoneal injection of an overdose of sodium pentobarbital at 7 or 28 days. The hearts were carefully removed and fixed with 10% neutral formalin by perfusion fixation. Fixed hearts were cut into three transverse sections from the ligated site of left coronary artery to the apex and embedded in paraffin. Sections (5 μm) were deparaffinized and stained with H&E (haematoxylin and eosin). Immunohistochemistry was performed as described previously [28]. The hearts were fixed overnight in methanol and embedded in paraffin. Sections (5 μm) were deparaffinized and incubated with a rat monoclonal antibody against murine CD31 (clone MEC13.3; BD PharMingen). Antibody binding was visualized using avidin–biotin and the Vector Red chromogenic substrate (Vector Laboratories), followed by counterstaining with haematoxylin. Capillaries were identified by positive staining for anti-CD31 antibody and morphology. The number of capillaries/mm^2 was counted in regions with transversely sectioned myocytes in the border (peri-infarct) zone. Three or four fields in the border zone per section were analysed for each mouse (a total of 10–12 fields/heart) at ×200 magnification [29,30]. Additional sections were counterstained with haematoxylin.

RT-PCR (reverse transcriptase-PCR)

Total RNA was prepared from both ischaemic and non-ischaemic myocardium using RNA Bee (Tel-Test) [31]. RT-PCR was performed as described previously [31]. First-strand cDNA was synthesized from 1 μg of total RNA, using oligo-$(dT)_{20}$ primer and MMLV (Moloney-murine-leukaemia virus)-derived reverse transcriptase (ReverTra Ace-α; Toyobo). A portion (1/20) of the reaction mixture was used as a template for PCR amplification. The mouse VEGF primers, 5′-GCGGGCTGCCTCGCAGTC-3′ (sense) and 5′-TCACCGCCTTGGCTTGTCAC-3′ (antisense), yielded products of 716 bp ($VEGF_{188}$), 644 bp ($VEGF_{164}$) and 512 bp ($VEGF_{120}$). PCR reactions were carried out for 30 cycles with 1 min of denaturation at 94 °C, 1 min of annealing at 65 °C and 1.5 min of extension at 72 °C, followed by 10 min of final extension. The mouse HGF primers, 5′-GCGGGCTGCCTCGCAGTC-3′ (sense) and 5′-TCACCGCCTTGGCTTGTCAC-3′ (antisense), yielded a product of 423 bp (HGF). PCR reactions were carried out for 30 cycles with 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C and 1.5 min of extension at 72 °C, followed by 10 min of final extension [32]. The human VEGF primers, 5′-GGACATCTTCCAGGAGTA-3′ (sense) and 5′-TGCAACGAGAGTCTGTGT-3′ (antisense), yielded products of 413 bp and 341 bp. PCR reactions were carried out for 30–35 cycles with 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C and 30 s of extension at 72 °C, followed by 10 min of final extension. The human HGF primers, 5′-ATGCTCATGGACCCTGGT-3′ (sense) and 5′-GCCTGGCAAGCTTCATTA-3′ (antisense), yielded a product of 423 bp (HGF). PCR reactions were carried out for 30 cycles with 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C and 1 min of extension at 72 °C, followed by 10 min of final extension.

Statistics

All values are means ± S.E.M. Multiple comparisons were performed by one-way ANOVA with Fisher's post-hoc comparison. Survival analysis was performed by Kaplan–Meier analysis and evaluated by log-rank test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of ONO-1301 on HGF and VEGF secretion

NHDFs were cultured on 48-well plates in the absence or presence of ONO-1301. ONO-1301 (1 μmol/l) increased the concentration of HGF and VEGF in the medium in a time-dependent manner, with a maximum response being obtained after 72 h (results not shown). Treatment with ONO-1301 (0.01–1 μmol/l) for 72 h increased HGF and VEGF concentrations in a dose-dependent manner (Figures 1A and 1B). Dibutyryl cAMP (0.5 mmol/l), an analogue of cAMP, and forskolin (1 μmol/l), an adenylate cyclase activator, mimicked the effect of ONO-1301 (Figures 1C and 1D). Rp-cAMP (0.5 mmol/l), a cAMP inhibitor, partially inhibited the effect of ONO-1301 (Figure 1E). These results suggested that cAMP, at least in part, mediated the effect of ONO-1301 in up-regulating HGF. When a neutralizing anti-HGF antibody was added, ONO-1301 failed to up-regulate VEGF expression in fibroblasts, suggesting that HGF may mediate ONO-1301-induced stimulation of VEGF production (Figure 1F). Furthermore, to investigate the effect of ONO-1301 on growth factor secretion in other cell types, the effect of ONO-1301 on HUVECs and HASMCs was examined. Up-regulation of HGF or VEGF was not be detected in ONO-1301-treated HUVECs as determined by ELISA (Figures 1G and 1H) or RT-PCR (results not shown). On the other hand, HASMCs produced HGF and VEGF abundantly, and ONO-1301 slightly, but significantly, up-regulated HGF secretion (Figure 1G).

Effect of ONO-1301 on *in vitro* angiogenesis

The angiogenic activity of ONO-1301 *in vitro* was evaluated using a two-dimensional co-culture system of HUVECs and NHDFs. ONO-1301 enhanced tube-like formation by HUVECs (Figure 2A). The angiogenic activity of ONO-1301 was comparable with that of VEGF or HGF. The angiogenic effect of ONO-1301 was reduced by administration of neutralizing anti-HGF or anti-VEGF antibodies. These findings suggested that the favourable effect of ONO-1301 on angiogenesis *in vitro* was mediated, at least in part, by up-regulation of HGF and VEGF.

Effect of ONO-1301 on endogenous growth factor expression in ischaemic myocardium

To deliver ONO-1301 locally and chronically, ONO-1301 was polymerized with PLGA to generate SR-ONO. The *in-vitro*-releasing test revealed that ONO-1301 was released continuously from SR-ONO within 12–14 days (Figure 3A). When the effect of PLGA and SR-ONO on growth factor secretion was tested, PLGA had no effect on HGF and VEGF expression (Figure 3B), whereas SR-ONO up-regulated secretion of both growth factors (Figure 3B). The angiogenic effect of ONO-1301 *in vivo* was evaluated by injecting SR-ONO into ischaemic myocardium immediately after ligation of the left anterior descending artery. No systemic effects, including those on blood pressure and heart rate, were observed following the local injection of SR-ONO into the heart. Expression of HGF and VEGF was analysed by RT-PCR in ischaemic and non-ischaemic areas at 7 days

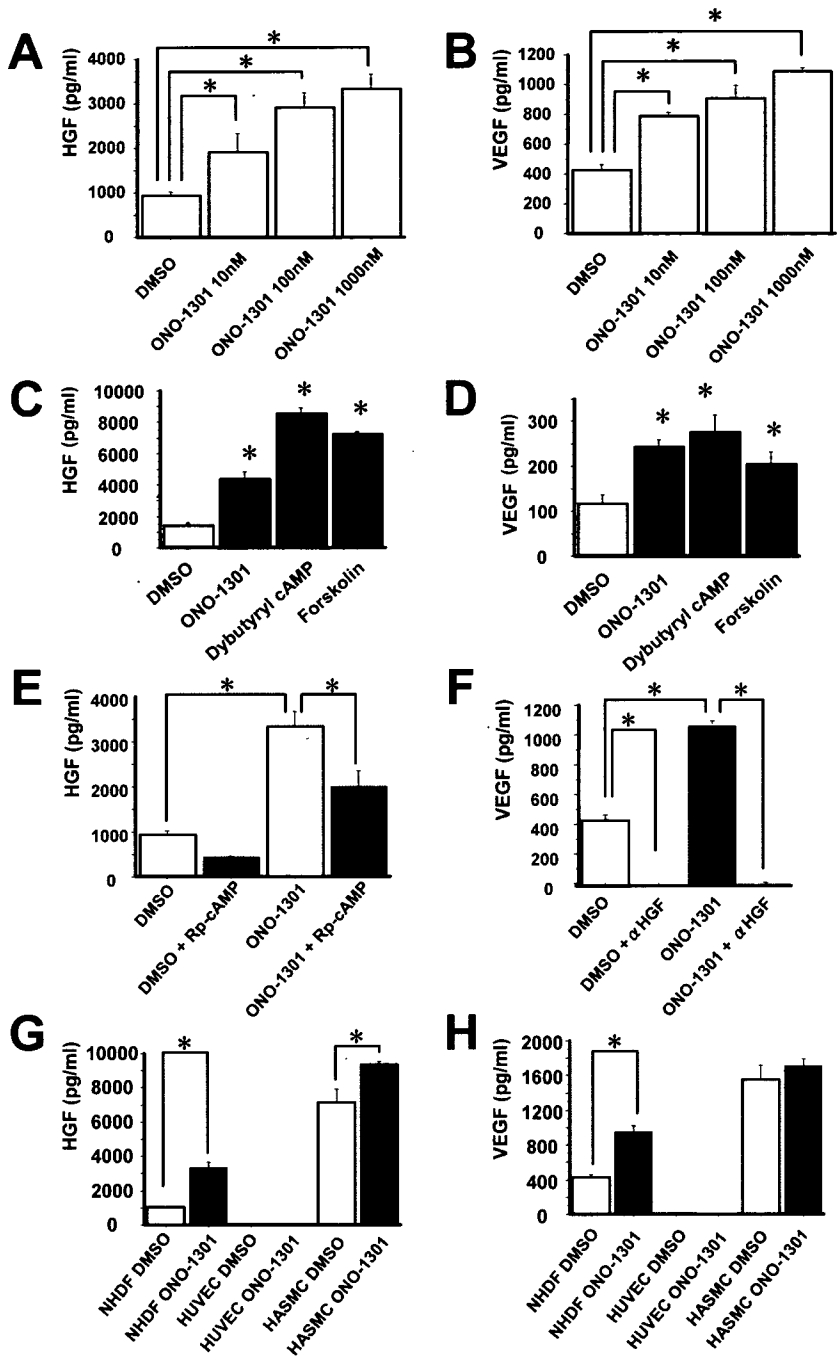

Figure 1 Effect of ONO-1301 on HGF and VEGF production

(A and B) NHDFs were cultured in the absence or presence of ONO-1301 (0.01–1 μmol/l) for 72 h. (C and D) NHDFs were cultured on a 48-well plate in the absence or presence of ONO-1301 (0.1 μmol/l), dibutyryl cAMP (0.5 mmol/l) and forskolin (1 μmol/l) for 72 h. (E) NHDFs were cultured with vehicle and ONO-1301 alone (1 μmol/l) or in combination with Rp-cAMP (0.5 nmol/l) for 72 h. (F) NHDFs were cultured with vehicle and ONO-1301 alone (1 μmol/l) or in combination with a neutralizing anti-HGF antibody (αHGF; 20 μg/ml) for 72 h. (G and H) HUVECs and HASMCs were cultured in the absence or presence of ONO-1301 (1 μmol/l) for 72 h. Following treatment, HGF (A, C, E and G) and VEGF (B, D, F and H) concentrations in the supernatants were determined by ELISA. In each panel, values are means $\pm$ S.E.M. of two or three replicate measurements. *$P < 0.05$.

after MI (Figure 3C). HGF mRNA was down-regulated in the ischaemic myocardium treated with vehicle, and SR-ONO partially restored the expression. VEGF expression was not detected in the non-ischaemic myocardium, but endogenous VEGF expression was slightly up-regulated in the ischaemic myocardium treated with vehicle. SR-ONO up-regulated VEGF expression further in ischaemic myocardium (Figure 3C).

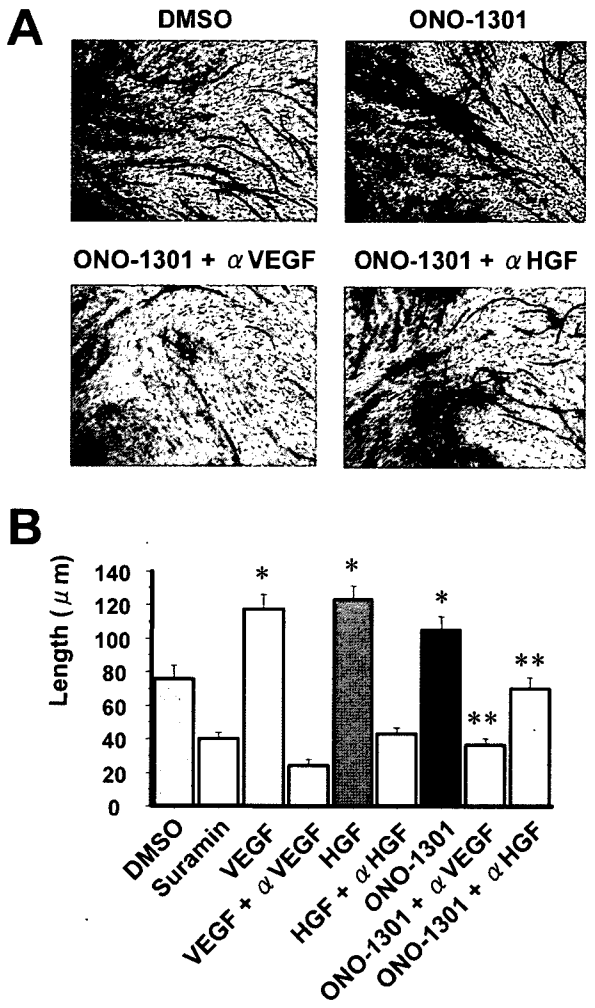

Figure 2 Promotion of *in vitro* tube formation by ONO-1301

(A) *In vitro* angiogenic activity of ONO-1301 was evaluated in the absence or presence of ONO-1301 (0.1 μmol/l), a neutralizing anti-(human HGF) antibody (αHGF; 10 μg/ml) and a neutralizing anti-(human VEGF) antibody (αVEGF; 5 μg/ml). At 11 days, cells were fixed with 10% formalin and stained using an anti-CD31 antibody. (B) Length of CD31-positive endothelial cells with tube-like structures was quantified in five random high-power fields ($\times$ 40 magnification) in a blinded fashion using an image analysing system ($n = 3$ for each group). Values are means $\pm$ S.E.M. $^*P < 0.05$ compared with control (DMSO); $^{**}P < 0.05$ compared with ONO-1301 alone.

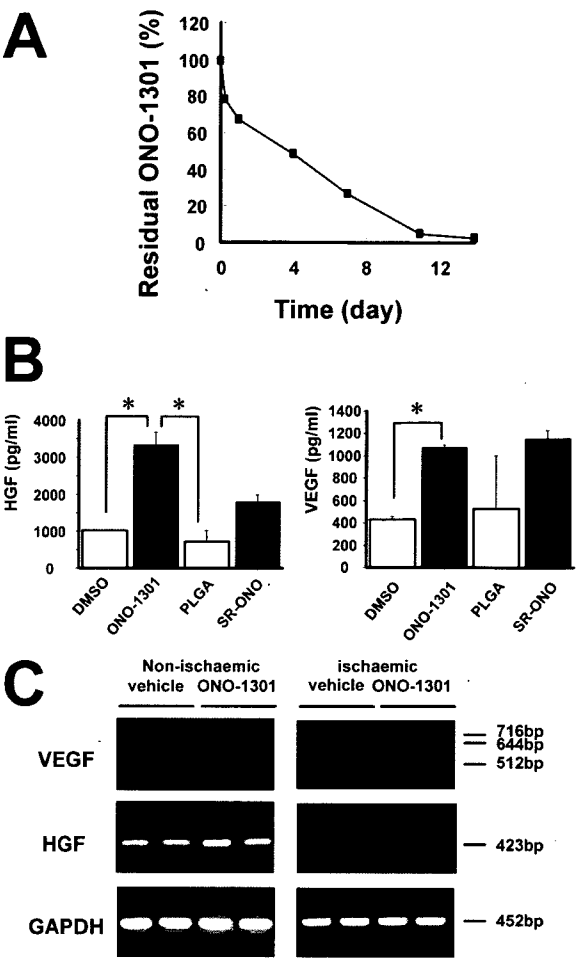

Figure 3 Up-regulation of HGF and VEGF expression by SR-ONO

(A) The releasing rate of the ONO-1301–PLGA complex in 0.2% Tween 80/sodium phosphate buffer was determined by measuring residual ONO-1301 in the pellets. (B) The effect of ONO-1301, PLGA and SR-ONO on growth factor secretion. ONO-1301 (1 μmol/l), PLGA (9 μg/ml) or SR-ONO (equivalent to 1 μmol/ml ONO-1301) were added to NHDFs. Supernatants were harvested after 72 h and HGF and VEGF concentrations were determined. $^*P < 0.05$. (C) Immediately after coronary ligation, 2 mg of SR-ONO was injected directly into the myocardium ($n = 5$). PLGA (2 mg) containing no ONO-1301 was injected as a control ($n = 5$). Total RNA was isolated at 7 days and RT-PCR was performed. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Effect of ONO-1301 on angiogenesis in ischaemic myocardium

Anti-CD31 immunostaining revealed that SR-ONO significantly increased capillary density in the ischaemic border zone at 7 days after MI (Figure 4A). The angiogenic effect of SR-ONO was partially abrogated by a neutralizing anti-(mouse VEGF) antibody (Figure 4B), suggesting that ONO-1301 augmented angiogenesis, at least in part, via the up-regulation of VEGF.

Effect of ONO-1301 on LV dilatation and survival after MI

LV function was evaluated by echocardiography at 0, 1 and 28 days after MI (Figure 5A). SR-ONO significantly attenuated LV remodelling at 28 days after MI, as determined by increased LVEDD and LVESD (Figure 5B). The survival rate up to 2 weeks after MI was significantly improved by SR-ONO ($P < 0.05$; Figure 6A). The beneficial effects of ONO-1301 were abrogated by administration of an anti-VEGF antibody (Figure 6B).

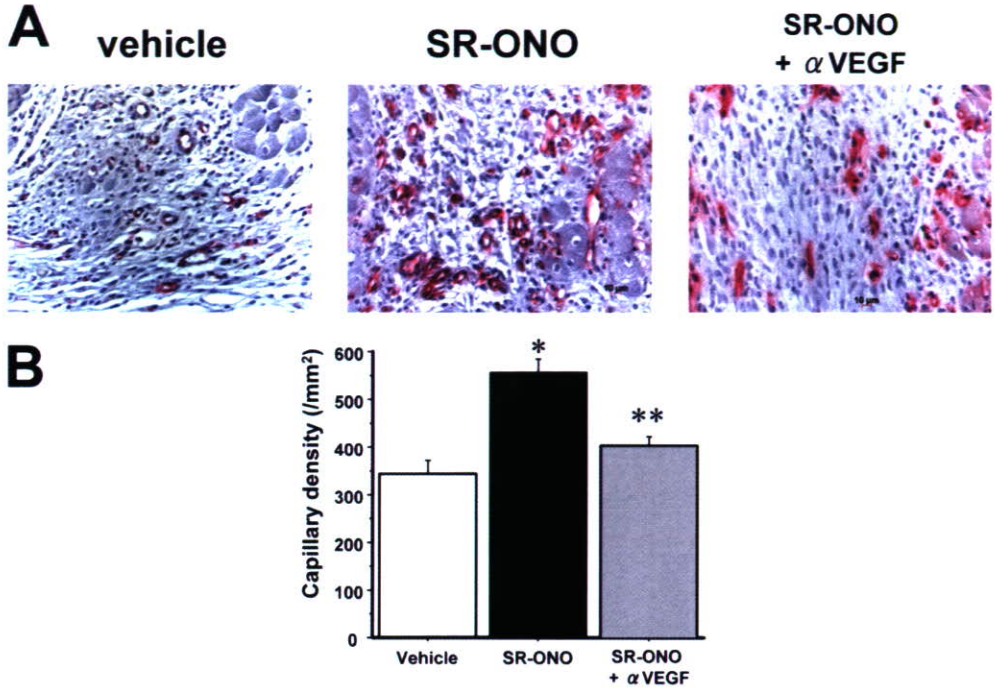

Figure 4 Increased capillary density at the border zone by ONO-1301 at 7 days after MI

(A) Capillary density at border zone at 7 days after treatment with vehicle and SR-ONO alone or in combination with a neutralizing anti-(human VEGF) antibody (SR-ONO + αVEGF). Endothelial cells were visualized using an anti-CD31 antibody and avidin–biotin and Vector Red chromogenic substrate, followed by counterstaining with haematoxylin. Magnification, $\times$ 200. (B) Capillaries were identified by positive staining for anti-CD31 antibody and morphology ($n = 5$ for each group). The number of capillaries/mm^2 was counted in regions with transversely sectioned myocytes in the border (peri-infarct) zone. Three or four fields in the border zone per section were analysed in each mouse (a total of approx. 10–12 fields/heart) at $\times$ 200 magnification. Values are means $\pm$ S.E.M. $^*P < 0.01$ compared with vehicle; $^{**}P < 0.05$ compared with SR-ONO.

DISCUSSION

In the present study, we have demonstrated that ONO-1301 induced the production of endogenous HGF and VEGF *in vitro* and *in vivo*. Injection of SR-ONO augmented angiogenesis in ischaemic myocardium and prevented LV enlargement. ONO-1301 also significantly improved survival after MI. The beneficial effects of ONO-1301 were abrogated by administration of a neutralizing antibody against VEGF.

Therapeutic angiogenesis has emerged as a promising strategy to treat ischaemic diseases. Administration of angiogenic growth factors as proteins or genes enhances collateral formation; however, previous studies have suggested that new vessels regenerated by the overexpression of a single growth factor are not fully functional [33,34], suggesting that a combination of several cytokines are required to regenerate physiological collateral arteries [7,35]. It has been reported that transplantation of bone marrow cells or circulating progenitor cells promotes new vessel formation in ischaemic tissues [3,36,37]. Bone-marrow-derived cells accumulate around growing collateral arteries with expression of several growth factors and chemokines [4,38]. It was hypothesized that bone-marrow-derived cells promote vascular growth not only by incorporating into vessel walls, but also by secreting several cytokines [4,5]. It is likely that an increase in the local concentration of growth factors at the site of ischaemia would favour collateral formation. Therefore stimulation of local residual cells to produce growth factors could be an alternative strategy to supply growth factors to the site of new vessel formation. In fact, it has been reported that cell transplantation stimulates ischaemic muscle to secrete angiogenic cytokines [39].

It has been shown that PGs regulate HGF expression from cultured cells [8,40]. HGF production was transcriptionally activated by PGE_1 and PGI_2, but only slightly by PGE_2 and PGD_2, in cultured human skin fibroblasts and smooth muscle cells [8,40]. In addition, administration of PGE_1 increased circulating HGF levels in patients with peripheral arterial disease [11]. It has been suggested that the generation of HGF is induced through a cAMP-mediated pathway [41], and a cAMP-responsive element consensus sequence has been located in the promoter region of the HGF gene [32]. HGF induces VEGF expression in smooth muscle cells [42,43] and keratinocytes [44], and Sakurai et al.

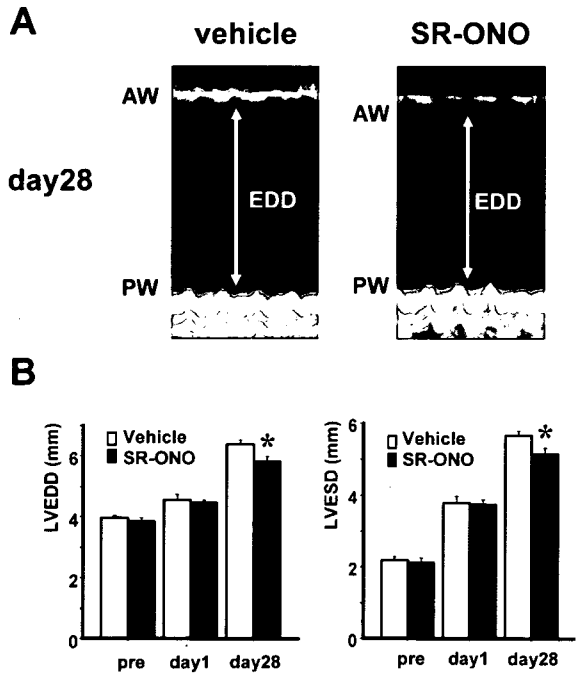

Figure 5 Amelioration of LV dilation by SR-ONO

Echocardiographic studies were performed under anaesthesia before surgery (pre) and at 1 and 28 days after MI ($n = 8$ for each group), and LVEDD and LVESD were measured. (A) Representative M-mode echocardiograms obtained from mice treated with vehicle and SR-ONO at 28 days after MI. AW, LV anterior wall; PW, LV posterior wall. (B) Quantification of LVEDD and LVESD in mice treated with vehicle or SR-ONO for up to 28 days after MI. Values are means ± S.E.M., $n = 8$ for each group. *$P < 0.05$ compared with vehicle at day 28.

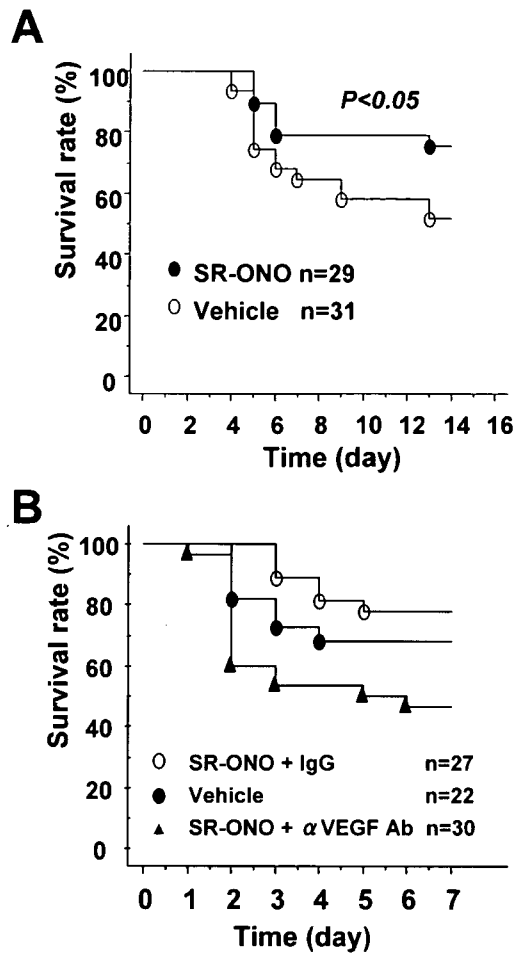

Figure 6 Kaplan–Meier survival analysis after MI in mice treated with vehicle or SR-ONO

MI was induced in 8–10-week-old male CD-1 mice and, immediately after ligation, vehicle (2 mg of PLGA microspheres) or SR-ONO (2 mg) was injected directly into the ischaemic myocardium. In (B), a neutralizing anti-(mouse VEGF) antibody (αVEGF Ab; 0.0025 μg/kg of body weight) or normal goat IgG (0.025 μg/kg of body weight) was administered intraperitoneally at 0, 3, 5 and 7 days. (A) The percentage of surviving mice treated with vehicle ($n = 31$) or SR-ONO ($n = 29$). (B) The percentage of surviving mice treated with vehicle ($n = 22$), SR-ONO + normal Ig ($n = 27$) or SR-ONO + αVEGF ($n = 30$). The difference between the groups was tested by the log-rank test.

[45] have shown that PGs stimulated retinal capillary pericyte proliferation with the induction of c-fos mRNA, phosphorylation of CREB (cAMP-response-element-binding protein) and increased expression of VEGF. Induction of VEGF by PGs was almost completely blocked by SQ22536, an adenylate cyclase inhibitor, suggesting that HGF production via the cAMP pathway is the major signalling pathway for the induction of VEGF mRNA by PGs. Thus local administration of PGs appears to effectively induce the production of HGF by local residual cells. HGF subsequently up-regulates VEGF. In the present study, ONO-1301, a synthetic non-prostanoid prostacyclin agonist, successfully up-regulated the expression of VEGF and HGF *in vitro* and *in vivo*. The effect of ONO-1301 was mimicked by a cAMP analogue or cAMP-elevating reagent and was inhibited by a cAMP inhibitor, suggesting that cAMP mediates the beneficial effect of ONO-1301, at least in part.

The prostanoid structure in prostacyclin and its analogues is subjected to metabolism by 15-hydroxyPG dehydrogenase. A phase I clinical trial investigating the oral administration of ONO-1301 has been stopped due to a high incidence of diarrhoea (Y. Sakai, unpublished work), possibly due to stimulation of gastrointestinal cells. Systemic administration of ONO-1301 also caused hypotension in mice and rats (Y. Sakai, unpublished work). To overcome these potential side effects caused by oral administration of ONO-1301, we generated SR-ONO with PLGA. PLGA can be gradually absorbed without tissue damage. We did not observe vasodilatation or hypotension, which was reported following systemic administration of a high dose of prostacyclin [46], its analogues or ONO-1301 (Y. Sakai, unpublished work).

Thus SR-ONO appears to enable ONO-1301 to be delivered locally and chronically at the site of ischaemia with minimum adverse systemic effects. For therapeutic use, it would be feasible to optimize the size of the microspheres, the time of drug release and the content of ONO-1301.

In ischaemic hearts, a slight up-regulation of VEGF was observed, which was significantly enhanced by SR-ONO. In contrast, HGF was constitutively expressed by non-ischaemic myocardium and was down-regulated in ischaemic myocardium, a finding consistent with previous studies showing that hypoxic treatment resulted in a significant decrease in local HGF production and a increase in VEGF expression in various cell types [47]. SR-ONO attenuated this ischaemic-induced down-regulation of HGF.

Results from the *in vitro* experiments suggested that growth factors were not induced in endothelial cells, although we could not perform immunohistochemistry to identify which cell types secreted growth factors in the ischaemic heart. It is plausible that growth factors secreted by neighbouring fibroblasts and smooth muscle cells stimulated residual endothelial cells in a paracrine manner.

ONO-1301 prevented LV dilatation with no significant effect on ejection fraction. The preventive effect of ONO-1301 on cardiac remodelling was relatively small when observed at 28 days after MI; however, ONO-1301 significantly increased the capillary density in the ischaemic heart. We also observed that ONO-1301 attenuated myocardial hypertrophy and fibrosis in the intact myocardium (results not shown). Further studies are required to evaluate the long-term effect of ONO-1301 on cardiac function in a chronic myocardial ischaemia model.

In summary, we have demonstrated that ONO-1301 stimulated fibroblasts to secrete angiogenic cytokines. SR-ONO up-regulated HGF and VEGF and promoted angiogenesis in ischaemic mouse hearts. Administration of synthetic small molecules is apparently less invasive than isolation and transplantation of endothelial progenitor cells and/or bone marrow cells. Repeated administration would be easy with a small synthetic molecule, in contrast with autologous bone marrow transplantation that requires bone marrow aspiration. Therefore ONO-1301 might have therapeutic potential in treating ischaemic diseases.

ACKNOWLEDGMENTS

This study was supported, in part, by grants from the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labor and Welfare of Japan. Y.S. is an employee of Ono Pharmaceutical Company Ltd.

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Received 24 October 2006/26 January 2007; accepted 31 January 2007
Published as Immediate Publication 31 January 2007, doi:10.1042/CS20060301