Ultrasound stimulation restores impaired neovascularization-related capacities of human circulating angiogenic cells

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Received 17 August 2011; revised 7 May 2012; accepted 23 May 2012; online publish-ahead-of-print 28 May 2012

Time for primary review: 22 days

Aims
Unsatisfactory effects of therapeutic angiogenesis in critical limb ischaemia may be ascribed to use of circulating angiogenic cells (CACs) derived from atherosclerotic patients with impaired neovascularization-related capacities. We tested whether ultrasound cell stimulation can restore the impaired capacities.

Methods and results
During culture of human peripheral blood-derived mononuclear cells for 4 days to achieve CACs, we stimulated the cells in culture daily with low-intensity pulsed ultrasound stimulation (LIPUS). Application of LIPUS to cells in culture derived from healthy volunteers augmented the generation and migration capacities of CACs, increased concentrations of angiopoietin 2 and nitrogen oxides in the culture medium, and increased the expression of phosphorylated-Akt and endothelial nitric oxide synthase in CACs on western blotting. Application of LIPUS to cells in culture derived from atherosclerotic patients also augmented the generation and migration capacities of CACs. Although neovascularization in the ischaemic hindlimb of athymic nude mice was impaired after intramuscular injection of CACs derived from atherosclerotic patients compared with that using CACs derived from healthy volunteers, LIPUS of the cells in culture derived from atherosclerotic patients restored the neovascularization capacities.

Conclusion
Therapeutic angiogenesis with LIPUS-pre-treated CACs may be a new strategy to rescue critical limb ischaemia in atherosclerotic patients.

Keywords
Circulating angiogenic cells • Neovascularization • Atherosclerotic risk factors • Ultrasound pre-treatment • Cell therapy

1. Introduction
Augmenting neovascularization is a therapeutic option to rescue tissues with ischaemia.1 Cell therapy with circulating angiogenic cells (CACs), which are considered to be present in bone marrow-derived mononuclear cells (BM-MNCs) or peripheral blood-derived mononuclear cells (PB-MNCs), may play a role in augmenting neovascularization.2–5 We first reported the clinical effects and safety of therapeutic angiogenesis stimulated by intramuscular injection of BM-MNCs for patients with critical limb ischaemia in a large-scale clinical trial.6,7 However, the effects are unsatisfactory. This may be ascribed to the use of CACs with impaired neovascularization-related capacities, which are observed in patients with risk factors of atherosclerosis.8,9 If we can restore the neovascularization-related capacity before injection of the CACs, desirable effects may be achieved.

Ultrasound, which is high- and low-frequency sound waves, has been used to transfer drugs or genes to target cells10,11 to stimulate angiogenesis for wound repair,12 and to deliver BM-MNCs13 or CACs14 to tissues with ischaemia for the purpose of augmenting neovascularization. Ikeda et al. reported that indirect low-intensity pulsed
ultrasound stimulation (LIPOS) of pluripotent mesenchymal cells augments cell differentiation to osteoblasts and chondroblasts. \(^{15}\) LIPOS was used as a non-invasive therapy to accelerate bone healing in rabbits. \(^{16}\) Takeuchi et al. have recently reported that LIPOS activates the phosphatidylinositol 3-\(\Omega\) kinase/Akt pathway and stimulates the growth of chondrocytes. \(^{17}\) Although the phosphatidylinositol 3-\(\Omega\) kinase/Akt pathway was reported to be important for the neovascularization-related capacities of CACs, \(^{18}\) there have been no reports on the effects of direct ultrasound stimulation of CACs on the neovascularization-related capacity. Accordingly, we tested whether non-invasive direct LIPOS can augment neovascularization-related capacities of human CACs obtained from healthy subjects and patients with atherosclerotic risk factors in vitro, and whether intramuscular injection of LIPOS-pre-treated CACs into the ischemic hindlimb of athymic nude mice could augment neovascularization.

2. Methods

2.1 CAC culture
A total of 30 mL of peripheral blood was collected from healthy volunteers or atherosclerotic cardiovascular disease (ACD) patients with more than two of the following atherosclerotic risk factors: age >65 years old, current smoking, hypertension, dyslipidemia, or diabetes. ACD was diagnosed by coronary angiography. No patients had acute symptoms of ACD or had ECG findings indicative of acute ischemia. This study conformed to the principles outlined in the Declaration of Helsinki and was approved by the Committee on the Ethics Review Board of Kurume University School of Medicine. The detailed protocol for CAC culture is described in the Supplementary Methods.

2.2 Ultrasound stimulation of cells in culture
We stimulated culture cells daily with rectangular pulsed ultrasound, which was generated by a sonoporator (Sonoporator KTAC-4000; NepaGenie, Chiba, Japan). \(^{11,19}\) The first ultrasound stimulation (US) was performed at 24 h after the beginning of cell culture, and then second and third US sessions were carried out 24 h apart (Figure 1A). US was set by a combination of various ultrasound-related variables: oscillation frequency of 1.011 MHz; burst frequency of 5.0 Hz; spatial average temporal by a combination of various ultrasound-related variables: oscillation frequency of 1.011 MHz; burst frequency of 5.0 Hz; spatial average temporal

2.3 Cell viability assay
We removed non-adherent cells from the culture plate after the first US. We detached adherent cells from the plate with trypsin (TrypLE\textsuperscript{\textregistered} Express; Invitrogen, Carlsbad, CA, USA) and stained them with 0.4% Trypan Blue solution. Trypan Blue-positive and -negative cells indicate dead and live cells, respectively. The numbers of Trypan Blue-positive and -negative cells were automatically counted with a cell counter (Countess\textsuperscript{\textregistered}; Cell Counter; Invitrogen, Carlsbad, CA, USA), and the viability ratio of adherent cells was calculated using the following formula: viability ratio (%) = \([\text{total number of Trypan Blue-negative cells/total number of Trypan Blue-positive cells plus Trypan Blue-negative cells}] \times 100.

2.4 Cell migration assay
We evaluated the migration capacity of CACs with the modified Boyden chamber system as described previously. \(^{21,22}\) Briefly, we filled the outside chamber with 750 \(\mu\)L of culture medium with stromal cell-derived factor-1a (SDF-1a: 100 ng/mL; R&D Systems Inc., Minneapolis, MN, USA). SDF-1a is a chemotactrant for CAC migration. The inside chamber, with a polyethylene terephthalate membrane basement (BD Biosciences, San Jose, CA, USA), was placed into the outside chamber. Pores of 8 \(\mu\)m diameter were randomly located on the surface of the membrane. A total of 1 \(\times\) 10\(^5\) cells in 500 \(\mu\)L of culture medium with 20% fetal bovine serum were poured onto the membrane and incubated for 24 h at 37°C. The cells transmigrated to the underside of the membrane into the lower chamber through the surface pores. After the incubation, transmigrated cells were fixed with 2% paraformaldehyde and then stained with 4',6-diamidino-2-phenylindole (DAPI). The DAPI-positive cells were counted in five randomly selected high-power fields under a fluorescence microscope.

2.5 Flow cytometric analysis
We assessed the phenotype of CACs by flow cytometric analysis. \(^{21,22}\) After detaching of CACs from the culture plate, we incubated the cells with antibodies to the following cell surface antigens: \(^{21–24}\) CD34 (PE-labelled mouse antibody; BD Biosciences), CD133 (PE-labelled mouse antibody; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), CD31 (PE-labelled mouse antibody; BD Biosciences), and KDR (biotin-conjugated mouse antibody Sigma, St. Louis, MO, USA; streptavidin–fluorescein isothiocyanate conjugate, BD Biosciences). We then analysed the expressions of the antigens using a flow cytometer (FACSCanto\textsuperscript{TMII}; Becton Dickinson, Franklin Lakes, NJ, USA).

2.6 Cell proliferation assay
We assessed cell proliferation of CACs by bromodeoxyuridine (BrdU) assay, At 2 h after a single session of LIPOS of the cells in culture, we fixed the adherent cells using special buffers (Fixation buffer, Perm Buffer III, and Stain Buffer; BD Pharmingen, Franklin Lakes, NJ, USA). We incubated the cells for 1 h at 37°C using DNase I solution (Sigma-Aldrich, St. Louis, MO, USA) to denature them and further incubated them with BrdU antibody (Alexa Fluor 488-labelled mouse antibody; BioLegend, San Diego, CA, USA) for 1 h at room temperature. We washed the cells using phosphate-buffered saline (PBS) and finally counterstained them with DAPI. The number of BrdU-positive CACs was manually counted under a fluorescence microscope. We calculated the percentage of BrdU-positive CACs using the following formula: percentage of BrdU-positive CACs = \(\frac{\text{total number of BrdU-positive cells}}{\text{total number of DAPI-positive cells}} \times 100.

2.7 Cell secretion assay
We measured the concentrations of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), SDF-1a, angiopoietin 1 (Ang-1), and angiopoietin 2 (Ang-2) in supplement-free culture medium with a high-sensitivity enzyme-linked immunosorbent assay kit (Amer sham Pharmacia Biotech, Piscataway, NJ, USA) to assess the secretion capacity of CACs. \(^{21,22}\) We also measured the concentration of nitrogen oxides (NOx) released from CACs in supplement-containing culture medium with a NO colorimetric assay kit (NO2/NO3 Assay Kit-C II (Colorimetric); Dojindo Laboratories, Kumamoto, Japan) based on Griess’s method. Both kits are commercially available, and we used them according to the manufacturers’ manuals.

2.8 Osteoblast differentiation assay
We washed LIPOS-pre-treated CACs on the well with PBS and incubated them for 24 h with RUNX2 mouse monoclonal antibody for osteoblast (Abcam, Cambridge, UK). We washed the cells again with PBS and then...
An illustration of the experimental protocol (A) and the system of ultrasound stimulation (US) of the cells in culture (B). Three sessions of US were carried out on the cells in culture and then circulating angiogenic cells (CACs) were collected on day 4. The collected CACs were used for in vitro or in vivo experiments. Ultrasound was applied from a circular ultrasound transducer for the cells in culture through a gas-permeable film in the base of the culture plate. A sterile gel pad was laid between the film and the transducer. (C) Representative microscopic images of CACs that were generated from peripheral blood-derived mononuclear cells. Adherent cells on the culture plate were stained with two fluorescent dyes, Dil-acLDL (red) and UEA-lectin (green). Cells stained with both colours were defined as CACs. (D) Representative microscopic images of CACs with or without US. CACs were characterized by Dil-acLDL (red dye) incorporation. More CACs were observed on the culture plate after US at 100 mW/cm² intensity for either 60 or 240 s exposure compared with the number of CACs after US with other conditions. (E) Pooled data of the number of generated healthy volunteer-derived CACs (H-CACs) [expressed per high-power field (HPF)]. US at 100 mW/cm² intensity for either 60 or 240 s exposure significantly increased the number of H-CACs compared with no US (**P < 0.01; *P < 0.05; n = 9, each). US at 300 mW/cm² intensity for either 60 or 240 s exposure significantly decreased the number of H-CACs compared with no US (***P < 0.001; n = 9, each).
incubated them with Alexa Fluor 488-conjugated secondary antibody (anti-mouse IgG; Invitrogen) for 1 h at room temperature. Positive-control cells for osteoblasts (NH141; Lonza Walkersville, Inc., Princeton, NJ, USA) were purchased commercially. Stained cells were observed under a fluorescence microscope.

2.9 Reactive oxygen species (ROS) production assay

We washed CACs and LIPUS-pre-treated CACs on the well with PBS. Next, we added a fluorogenic probe for measuring cellular oxidative stress (20 μM; CellROX™ Deep Red Reagent; Invitrogen) and then incubated them for 30 min to show ROS in the cells. ROS-positive control CACs were prepared by incubating CACs with menadione (100 μM; Sigma-Aldrich) for 1 h. Menadione, a soluble form of vitamin K, is used to produce ROS in cells. Finally, we stained CACs with DAPI and observed ROS-positive CACs under the fluorescence microscope.

2.10 Western blot analysis

The detailed protocol is described in the Supplementary Methods.

2.11 Animal model of hindlimb ischaemia

We assessed the neovascularization capacity of CACs in vivo with a unilateral hindlimb ischaemia animal model using 8- to 12-week-old athymic nude mice (BALB/cA-nu/nu; CLEA Japan Inc., Tokyo, Japan). The details were described in our previous publications. Briefly, the proximal portion of the femoral artery and the distal portion of the saphenous artery were occluded using an electrical coagulator (Vetroson, V-10 Bi-polar, Electrosurgical Unit, Summit Hill Laboratories, Tinton Falles, NJ, USA), and the overlying skin was closed using nylon sutures. At 7 days after the surgery, we injected a total of 30 μL PBS with or without CACs (5 × 10^3 cells per mouse) at five equally spaced points of the adductor muscle. The investigation conformed to the Care and Use of Laboratory Animals published by the US National Institutes of Health. In addition, the study protocol was approved by the Institutional Animal Care and Use Committee of Kurume University School of Medicine. All procedures on the mice were performed under general anaesthesia with 100% oxygen for maintenance) using an animal anaesthesia machine (model TK-5, Bio Machinery, Chiba, Japan). The depth of anaesthesia was monitored by the toe-pinch reflex test.

2.12 Measurement of limb perfusion

At 14 days after the intramuscular injection of PBS with or without CACs, we determined the blood flow of both limbs of the mice with a laser Doppler blood flow imager (Laser Doppler Perfusion Imager System, MoorLDF™, Mark 2; Moor Instruments Ltd., Devon, UK). Before scanning the blood flow, mice were placed on a heating pad at 37°C to minimize variations in temperature. The mean laser Doppler flux was analysed on both limbs with software supplied by the manufacturer. To avoid the effects of the variables of ambient light and temperature, calculated perfusion is expressed as the ischaemic/non-ischaemic hindlimb blood flow ratio.22,25

2.13 Histology

For histological and morphological analysis of the ischaemic hindlimb, we analysed the capillary density in 5-μm-thick frozen sections of ischaemic and non-ischaemic adductor muscles of the mice at 14 days after the intramuscular injection of PBS with or without CACs. Mice were killed by cervical dislocation after induction of general anaesthesia with isoflurane in oxygen, and then the tissues were harvested. Capillary endothelial cells were stained for CD31 monoclonal antibody (fluorescein isothiocyanate labelled; BD Biosciences).22 The numbers of CD31-positive capillaries (<20 μm) and myofibres in 10 random microscopic high-power fields (×200 magnification) of the same traverse sections were counted with a fluorescence/light microscope (BX50; Olympus). We divided the number of capillaries by the number of myofibres to calculate the capillary density.

2.14 Statistical analysis

All values are expressed as means ± SEM. Results of CACs and US-pre-treated CACs in Figure 3D and E were compared and analysed with Student’s unpaired t-test. Other statistical analyses were performed by one-way ANOVA with Tukey’s post hoc test. Values of *P* < 0.05 were considered statistically significant. All analyses were performed with SPSS Inc., Chicago, IL, USA.

3. Results

3.1 Studies of CACs obtained from healthy volunteers

3.1.1 Number of CACs after US

Most of the adherent cells on the culture plate were stained with Dil-acetylated LDL (acLDL) and Ulex europaeus (UEA)-lectin (Figure 1C), indicating successful generation of CACs.22,25 US of 30 mW/cm² intensity for 15, 60, or 240 s exposure did not alter the number of healthy volunteer-derived CACs (H-CACs). However, US of 100 mW/cm² intensity for either 60 or 240 s exposure significantly increased the number of H-CACs. In contrast, US of 300 mW/cm² intensity for 15, 60, or 240 s exposure destroyed many cells and markedly decreased the number of H-CACs (Figure 1D and E).

3.1.2 Temperature of culture medium during US

To check whether US affects the temperature of the culture medium, we monitored the temperature during US. US of 30 and 100 mW/cm² intensity did not change the temperature until 420 s (upper panel in Figure 2A; data for US of 30 mW/cm² intensity are not shown). However, US of 300 mW/cm² intensity significantly increased the temperature after 240 s (lower panel in Figure 2A).

3.1.3 Cell viability after US

The viability ratio of healthy volunteer-derived culture cells after US of 100 mW/cm² intensity for 240 s exposure was similar to that of the cells after no US. However, US of 300 mW/cm² intensity for 240 s exposure significantly decreased the ratio (Figure 2B).

3.1.4 Migration capacity of H-CACs after US

As US of 100 mW/cm² intensity for either 60 or 240 s exposure significantly augmented the generation capacity of H-CACs (Figure 1E), we compared the migration capacity of H-CACs after US of 100 mW/cm² intensity between 60 and 240 s exposure. The number of migrated CACs was significantly increased by US of 100 mW/cm² intensity for 240 s but not for 60 s exposure (Figure 2C). On the basis of these results, we adopted non-invasive LIPUS of 100 mW/cm² intensity for 240 s exposure for the following experiments.

3.1.5 Phenotype of H-CACs after LIPUS

In the flow cytometric analysis, the expressions of both haematopoietic cell markers CD34 and CD133 and endothelial cell markers CD31 and KDR were similar on the surfaces of H-CACs and LIPUS-pre-treated H-CACs (Figure 2D). This result indicated that LIPUS did not augment the differentiation of the culture cells into CACs.
Figure 2  (A) Representative time course of temperature of the culture medium during US. US at 100 mW/cm² intensity did not change the temperature of the medium. US at 300 mW/cm² intensity began to increase the temperature after 60 s, and the temperature at 240 s was significantly higher than that at 240 s after US at 100 mW/cm² intensity (1.36 ± 0.1 vs. 0.2 ± 0.13°C increase in the temperature; *p < 0.05; n = 5, each). (B) The viability ratio of cells in culture after US. The ratio was significantly lower for the cells after US at 300 mW/cm² intensity (filled bar) than that after no US (open bar) or US at 100 mW/cm² intensity (grey bar; *p < 0.05; n = 5, each). (C) The number of migrated CACs for SDF-1α. The number was significantly greater for CACs after US at 100 mW/cm² intensity for 240 s (filled bar), but not for 60 s exposure (grey bar) than those after no US (open bar; *p < 0.01; n = 5, each). (D) Representative histograms of CACs and low-intensity pulsed ultrasound stimulation (LIPUS)-pre-treated CACs in the FACS analysis. The grey histogram corresponds to the isotype-matched IgG control. The patterns of the histograms for CD34, CD133, CD31, and KDR were similar between CACs (black histograms) and LIPUS-pre-treated CACs (purple-red histograms). The same expression patterns were observed in five experiments. (E) Representative fluorescence photographs of BrdU-positive and BrdU-negative CACs on the culture plate at 2 h after a single session of LIPUS (upper panels) and bar graph for pooled data of the percentage of BrdU-positive H-CACs on the plate (lower panel). Yellowish green colours and blue colours indicate BrdU and DAPI in the nuclei of CACs, respectively. The percentage was greater for LIPUS-pre-treated CACs than that for LIPUS-untreated CACs (*p < 0.001; n = 5).
3.1.6 Proliferation capacity of H-CACs after LIPUS
The number of BrdU-positive H-CACs was greater at 2 h after a single session of LIPUS than at 2 h after no LIPUS (Figure 2E). This result indicated that LIPUS augmented the proliferation of H-CACs.

3.1.7 Secretion capacity of H-CACs after LIPUS
We measured the concentrations of VEGF, b-FGF, SDF-1α, and Ang-1, and Ang-2 in the supplement-free culture medium of H-CACs. Only the concentration of Ang-2 was significantly increased after LIPUS (Table 1).

3.1.8 Cell signalling after LIPUS
In the western blot analysis, LIPUS increased the expression of endothelial nitric oxide synthase (eNOS) of H-CACs from 1 to 6 h in a time-dependent manner (Figure 3A). The expression of eNOS was significantly higher at 6 h after a single session of LIPUS (Figure 3A) and at 24 h after three sessions of LIPUS than in cells with no LIPUS (Figure 3B). LIPUS increased the expression of p-Akt in H-CACs after from 0.5 to 1 h; thereafter, the p-Akt expression gradually decreased (Figure 3C).

3.1.9 NOx concentration in the culture medium after LIPUS
The concentration of NOx in the culture medium gradually increased after from 0.5 to 1 h; thereafter, the p-Akt expression gradually decreased (Figure 3D).

3.2 Studies of CACs obtained from atherosclerotic patients
3.2.1 Number of CACs after LIPUS
The number of atherosclerotic patient-derived CACs (P-CACs) was significantly lower than that of H-CACs, indicating impaired generation capacity of P-CACs. LIPUS restored the generation capacity of P-CACs (Figure 4A).

3.2.2 Migration capacity of P-CACs after LIPUS
The number of migrated CACs for SDF-1α was significantly increased after LIPUS (Figure 4B) and migration capacities of H-CACs (Figure 4C). Taken together, in vitro experiments showed that LIPUS augmented the neovascularization-related capacity of CACs obtained from not only healthy subjects but also from atherosclerotic patients. Accordingly, we examined the effects of LIPUS-pre-treated CACs in an animal ischaemic model.

3.2.3 Safety of P-CACs after LIPUS
LIPUS-pre-treated P-CACs did not show cell expression characteristics of osteoblasts (Figure 4D). In addition, LIPUS-pre-treated P-CACs did not produce ROS (Figure 4E).

3.3 In vivo neovascularization capacity of H- and P-CACs after LIPUS
Intramuscular injection of H-CACs into the ischaemic hindlimb of athymic nude mice brought greater blood perfusion to the treated limb after 14 days than the injection of PBS (α vs. β in Figure 5A). The capillary density in the ischaemic hindlimb was greater at 14 days after the injection of H-CACs than after the injection of PBS (α vs. β in Figure 5B). Furthermore, the injection of LIPUS-pre-treated H-CACs increased the blood perfusion and capillary density, suggesting an augmented in vivo neovascularization capacity of the H-CACs. Intramuscular injection of P-CACs did not change blood perfusion and capillary density in the treated limb after 14 days compared with the injection of PBS. The blood flow ratio and capillary density at 14 days were lower for the injection of P-CACs than for the injection of H-CACs (β vs. d in Figure 5A and B). These results suggest that the in vivo neovascularization capacity of P-CACs was impaired compared with that of H-CACs. However, the injection of LIPUS-pre-treated P-CACs increased the blood perfusion, blood flow ratio, and capillary density to levels comparable to those following treatment with H-CACs (Figure 5A and B), suggesting a restored in vivo neovascularization capacity of the P-CACs.

4. Discussion
4.1 Effects of LIPUS on CAC functions in vitro
It was reported that LIPUS augmented the differentiation capacity of pluripotent mesenchymal cells to osteoblasts and chondroblasts in vivo.15 CACs contribute to neovascularization through their various capacities as follows: chemotaxis and adhesion to mature endothelial cells; migration and invasion of the intracellular space in adjacent endothelial cells; and secretion of cytokines to stimulate the sprouting of new capillaries from pre-existing arteries.21,24 In the present study, we examined whether LIPUS could augment these neovascularization-related capacities of CACs in vitro. We tested several experimental conditions of US to find the most appropriate condition. As described in the Methods, we fixed the oscillation frequency, burst frequency, and duty cycle on the basis of our previous studies.10,11,19,20 In the present study, we altered only the intensity and exposure time. For generation capacity, US at 30 mW/cm2 intensity was not effective and US at 300 mW/cm 2 intensity destroyed H-CACs (Figure 1E). US at 300 mW/cm2 intensity increased the temperature of the culture medium (Figure 2A) and decreased the viability of the cells (Figure 2B). In contrast, US at 100 mW/cm2 intensity for 240 s exposure augmented generation (Figure 1E) and migration capacities of H-CACs (Figure 2C), while maintaining cell viability (Figure 2B). Thus, the best condition of LIPUS was the 100 mW/cm2 intensity for 240 s. LIPUS at 100 mW/cm2 intensity for 240 s augmented the proliferation of H-CACs (Figure 2E), but not the differentiation of the culture cells into

| Table 1 Concentrations of neovascularization-related cytokines in culture medium |
|---------------------------------|---------------------|---------------------|---------------------|---------------------|
|                                  | LIPUS (−) (n = 6)  | LIPUS (+) (n = 6)  | P value             |
|---------------------------------|---------------------|---------------------|---------------------|---------------------|
| VEGF (× 10 pg/mL)               | 5.3 ± 1.1           | 6.1 ± 2.0           | n.s.                |
| b-FGF (× 10 pg/mL)              | 51.0 ± 26.6         | 55.5 ± 22.2         | n.s.                |
| SDF-1α (× 10 pg/mL)             | 16.8 ± 7.4          | 17.5 ± 10.0         | n.s.                |
| Angiopoietin 1 (× 10 pg/mL)     | 158.7 ± 26.2        | 181.7 ± 20.0        | n.s.                |
| Angiopoietin 2 (× 10 pg/mL)     | 92.4 ± 3.0          | 119.4 ± 5.9         | <0.05               |

Abbreviations: b-FGF, basic fibroblast growth factor; LIPUS, low-intensity pulsed ultrasound stimulation; n.s., not significant; SDF-1α, stromal cell-derived factor-1α; VEGF, vascular endothelial growth factor.
Figure 3 (A) Time course of the expression of endothelial nitric oxide synthase (eNOS) in H-CACs after LIPUS. In the representative western blotting photograph (upper panel), the expression of eNOS began to increase after 1 h and then increased in a time-dependent manner until 6 h. In the bar graph for pooled data of the eNOS/GAPDH expression ratio (lower panel), the ratio was significantly higher at 6 h after LIPUS than that before LIPUS (*P < 0.05; n = 5). (B) A western blot photograph of the expression of eNOS in H-CACs. Increased expression of eNOS was observed in H-CACs at 24 h after three sessions of LIPUS (upper panel). In the bar graph for pooled data of the eNOS/GAPDH expression ratio (lower panel), the ratio was significantly greater for LIPUS-pre-treated CACs than that for LIPUS-untreated CACs (*P < 0.05; n = 5). (C) Time course of the expressions of Akt and p-Akt in H-CACs after LIPUS. In the representative western blotting photograph (upper panel), the expression of p-Akt began to increase after 0.5 h and reached its peak level at 1 h. In the bar graph for pooled data of the p-Akt/Akt expression ratio (lower panel), the ratio was significantly higher at 1 h than that before LIPUS (*P < 0.05; n = 5). (D) Time course of the concentration of NOx in the culture medium. The open and filled bars in the bar graph indicate the percentage changes of the concentration after no LIPUS and after LIPUS, respectively. The percentage changes were significantly increased at 2, 3, and 6 h after LIPUS compared with those with no LIPUS (*P < 0.05, each; n = 5, each). (E) Concentration of NOx in the culture medium. The open and filled bars indicate the concentration after no LIPUS and three sessions of LIPUS, respectively. The concentration was significantly increased at 24 h after three sessions of LIPUS compared with that for no LIPUS (*P < 0.005; n = 5, each).
H-CACs (Figure 2D). The augmented proliferation capacity of LIPUS-pre-treated H-CACs might have contributed to the increase in CAC numbers shown in Figure 1E. Although we have no data on why LIPUS increased proliferation of CACs in this study, it has been reported that LIPUS stimulated the proliferation of chondrocytes via the integrin/phosphatidylinositol 3-OH kinase/Akt pathway.\textsuperscript{17} Thus, LIPUS-stimulated proliferation of CACs might be brought about by the activation of Akt (Figure 3C).

We examined the mechanisms by which LIPUS augmented neovascularization-related capacities of CACs. We focused on nitric oxide, given the following previous findings. First, Murohara et al. reported that NO was a key regulator of endothelial cell migration.\textsuperscript{26} Second, we reported that eNOS was a key player to augment migration and neovascularization capacities of CACs.\textsuperscript{25} Third, Altland et al. reported that low-intensity ultrasound irradiation increased the activity of eNOS in endothelial cells.\textsuperscript{27} Accordingly, we tested whether LIPUS could increase the activity of eNOS in CACs and NO secretion from CACs. In the western blot analysis, LIPUS increased the expression of eNOS of CACs and increased NOx concentration in the culture medium in a time-dependent manner (Figure 3A and D, respectively). Up-regulation of eNOS and elevation of NOx in LIPUS-pre-treated CACs were observed at 24 h after three sessions of LIPUS (Figure 3B and E, respectively). These results might have suggested that the up-regulation of eNOS and NO after LIPUS contributed to increased blood flow and capillary density in ischaemic limbs of mice. However, this was one of the possibilities for the mechanisms of augmented neovascularization. As eNOS is activated by p-Akt,\textsuperscript{28} we examined whether LIPUS increased the expression of p-Akt by CACs. LIPUS did increase the expression of p-Akt, and the peak of p-Akt expression preceded that of the expression of eNOS (Figure 3C). These results may suggest that LIPUS activates the Akt–eNOS signalling with increased NO secretion from CACs, which may be responsible for the augmented neovascularization-related capacities of CACs. The time course of Akt activation (Figure 3C) did not exactly correspond to that of the concentration of NOx secreted in the medium (Figure 3D). As we did not examine the time course of eNOS activity, we do not know the reason for this time lag. There are several possible reasons, as follows. First, not only Akt but also protein kinase A, protein kinase C, and extracellular-signal-regulated kinase activate eNOS;\textsuperscript{29,30} therefore, the delayed increase in NOx concentration may have reflected various time courses of activation.

**Figure 4** (A) The number of generated CACs. Although the number was significantly smaller for atherosclerotic patient-derived CACs (P-CACs; grey bar) than for H-CACs (open bar; *\textsuperscript{p} < 0.05; n = 5, each), LIPUS significantly increased the number of P-CACs (grey vs. filled bar; \textsuperscript{*p} < 0.05; n = 5, each). (B) The number of migrated CACs for SDF-1\textalpha. Although the number was significantly smaller for P-CACs (grey bar) than for H-CACs (open bar; \textsuperscript{*p} < 0.05; n = 5, each), LIPUS significantly increased the number of migrated P-CACs (grey vs. filled bar; \textsuperscript{*p} < 0.05; n = 5, each). (C) Representative photographs of CACs and osteoblasts. Upper and lower panels are light and fluorescence microscopic photographs, respectively. Osteoblasts expressed RUNX2 (green), which is a transcription factor associated with osteoblasts. CACs and LIPUS-pre-treated CACs did not express RUNX2, indicating no differentiation to osteoblasts. Similar findings were obtained in three individual experiments. Scale bar represents 100 \textmu m. (D) Production of reactive oxygen species (ROS) in CACs. DAPI stained the nuclei of CACs (blue). Although menadione (MND)-pre-treated CACs were positive for ROS production (red), CACs and LIPUS-pre-treated CACs were negative for ROS. Similar findings were achieved in three individual experiments. Scale bar represents 100 \textmu m.
Figure 5 (A) Representative laser Doppler blood flow images of both limbs of athymic nude mice (upper panel) and bar graph for pooled data of the ischaemic/non-ischaemic hindlimb blood flow ratio (lower panel) at 14 days after intramuscular injection of phosphate-buffered saline (PBS) or CACs into the right ischaemic limb. Black arrowheads on the images indicate ischaemic limbs. Red to white colour and dark blue colour on the image indicate high and low perfusion signals, respectively. (B) Representative fluorescence microscopic photographs of CD31-positive endothelial capillaries in the ischaemic limb (upper panel) and bar graphs for pooled data of the capillary density at 14 days after the injection of PBS or CACs into the ischaemic limb. The CD31-positive endothelial capillaries were stained green. Each image or photograph corresponds to the graph depicted immediately below. The injection of H-CACs increased the perfusion signal in the ischaemic hindlimb, the blood flow ratio, the number of CD31-positive endothelial capillaries, and the capillary density compared with the injection of PBS (bar graph a vs. b; *P < 0.05, §§P < 0.0001; n = 8, each). The injection of LIPUS-pre-treated H-CACs brought further increases (bar graph b vs. c; *P < 0.05, §P < 0.05; n = 8, each). The signal, ratio, number, and density after the injection of P-CACs were similar and inferior to those after the injection of PBS (bar graph a vs. d; n = 8, each) and H-CACs (bar graph b vs. d; *P < 0.05, §P < 0.05; n = 8, each). The injection of LIPUS-pre-treated P-CACs increased these indices compared with the injection of P-CACs (bar graph d vs. e; **P < 0.005, §P < 0.05; n = 8, each). The levels of signal, ratio, number, and density after the injection of LIPUS-pre-treated P-CACs were comparable to those after the injection of H-CACs (bar graph b vs. e; P = n.s.).
of these kinases. Second, it is possible that there might have been a time lag between eNOS activation, NOx production, and secretion. LIPUS increased the concentration of not only NOx but also Ang-2 in the culture medium of CACs. Ang-2 is a ligand of Tie-2, which is a major endothelium-specific receptor tyrosine kinase that plays a role in angiogenesis. Kim et al. reported that Ang-2 promoted late-outgrowth endothelial progenitor cell migration. Hildbrand et al. reported that Ang-2 regulated the development of endothelial cells from undifferentiated circulating precursors. Thus, the Ang-2 secreted from CACs may have contributed to augmented generation and migration capacities of CACs.

In the present study, the generation and migration capacities of CACs were impaired in subjects with atherosclerotic risk factors (Figure 4A and B). Although there have been pharmacological approaches to restore impaired neovascularization-related capacities of P-CACs, this is the first report on a physiological approach to restore impaired neovascularization-related capacities of P-CACs. LIPUS restored the impaired generation and migration capacities of P-CACs. Heeschen et al. reported that the in vitro migration capacity of CACs was closely correlated with the in vivo neovascularization capacity of the cells, suggesting that the in vitro migration capacity of CACs is a parameter to evaluate the degree of in vivo neovascularization capacity. Accordingly, we examined in the following experiment whether the in vivo neovascularization capacity of P-CACs was impaired and whether LIPUS restored it in an animal model of hindlimb ischaemia.

4.2 In vivo effects of LIPUS-pre-treated CACs

As we and others have reported, exogenously administered endothelial progenitor cells not only enhanced blood flow of the ischaemic limb but also increased capillary density, compared with PBS. It was also reported that the neovascularization by CACs obtained from patients with atherosclerotic risk factors was impaired. We obtained similar results in the present study. In accordance with the in vitro results, LIPUS-pre-treated H-CACs augmented in vivo neovascularization. Although the application of LIPUS to H-CACs looked like yielding the best results, this was not the case. The laser Doppler blood flow ratio and capillary density were statistically comparable between LIPUS-pre-treated H-CACs and LIPUS-pre-treated P-CACs (Figure 5A and B). Nonetheless, we need to discuss why LIPUS of H-CACs augmented in vivo neovascularization. There are several possible explanations. As we showed in vitro, LIPUS augmented Akt-eNOS signalling and increased NO and Ang-2 secretions from H-CACs, suggesting that these mechanisms may play a role even in vivo. Therefore, the capacity of LIPUS-untreated H-CACs may not yet be optimal for neovascularization. In fact, it has been reported that LIPUS of healthy pluripotent mesenchymal cells augmented cellular differentiation to osteoblasts and chondroblasts. Moreover, LIPUS of healthy chondrocytes activated the phosphatidylinositol 3-OH kinase/Akt pathway and stimulated the growth. These results may suggest that LIPUS further augments the functions of healthy cells.

LIPUS-pre-treated P-CACs restored the blood flow and capillary density to the level of H-CACs or LIPUS-pre-treated H-CACs. Although the in vivo mechanisms for augmented neovascularization by LIPUS-pre-treated P-CACs were unclear, our in vitro findings may suggest involvement of CAC-secreted NO and/or Ang-2. It is possible that CAC-secreted NO mediated vasodilatation of pre-existing and newly formed vessels and contributed to the increase of blood flow in the ischaemic limb. Although the in vivo mechanisms for augmented neovascularization were certainly multi-factorial and unclear, and we have no data to explain how the injection of in vitro isolated CACs augmented neovascularization in vivo 14 days later, several mechanisms are considered here. First, LIPUS-pre-treated CACs might have migrated more to the ischaemic limb, proliferated more, and produced NO and Ang-2 more than LIPUS-untreated CACs after the intramuscular injection. Second, it is well known that once blood flow to the ischaemic tissue is increased by NO, a favourable cycle for neovascularization is initiated, as follows: (i) NO induces endothelial cell proliferation and pericyte relaxation followed by capillary enlargement; (ii) the capillary enlargement induces increases of capillary blood flow, shear stress, and wall strain; (iii) the increases stimulate arteriogenesis and angiogenesis sprouting from the capillary and induce capillary arteriализation followed by vessel growth; and (iv) the stimulated angiogenesis decreases peripheral resistance and thereby increases blood flow in up-stream collateral arteries. Moreover, Moncada et al. reported that shear stress stimulated NO synthesis in endothelial cells. This might suggest that the increase of shear stress in the ischaemic limbs of the mice after the injection of CACs stimulated NO synthesis by the pre-existing endothelial cells of the limbs and thereby provided much more NO to the limbs. Such synergistically increased NO might further augment the blood flow-mediated arteriogenesis, angiogenesis, and collateral growth for the limbs after the injection of LIPUS-pre-treated CACs. Thus, the augmented neovascularization capacities observed in vitro might have worked in concert, and the effect might have persisted for 14 days. In fact, we previously reported that a single intravenous injection of CACs with the pharmacologically augmented capacity of NO synthesis gradually increased blood flow and capillary density of the ischaemic limbs of mice up to 14 days after the injection. Whatever the mechanisms are, LIPUS pre-treatment of CACs obtained from patients with atherosclerotic risk factors would be a promising strategy for treatment of peripheral artery disease.

It was reported that LIPUS augmented the differentiation capacity of pluripotent mesenchymal cells to osteoblasts. In order to examine the side effects of LIPUS, we investigated in vitro whether LIPUS induced differentiation of CACs to osteoblasts and whether LIPUS-pre-treated CACs produced ROS. LIPUS did not induced differentiation of CACs to osteoblasts (Figure 4C). and LIPUS-pre-treated CACs did not produce ROS (Figure 4D); however, these results do not necessarily preclude potential side effects of LIPUS-pre-treated CACs in vivo.

4.3 Study limitations

There were several limitations in this study. First, we generated CACs from PB-MNCs but not BM-MNCs. If we were to perform LIPUS for late-outgrowth endothelial progenitor cells or for CACs generated from BM-MNCs, different results might be shown. Second, we assessed the in vivo neovascularization effects by LIPUS-pre-treated P-CACs in non-atherosclerotic mice. It would be interesting to assess the effects of LIPUS-pre-treated P-CACs in atherosclerotic mice. However, as the mouse is highly resistant to atherosclerosis, this issue was not addressed in the present study. Third, although we assessed neovascularization of ischaemic hindlimbs of mice with a laser Doppler flowmeter, more quantitative measures of microvascular perfusion and microvascular blood volume by other
modalities, such as magnetic resonance imaging or contrast-enhanced ultrasonography, may be desirable to complement our results.

In the present study, the generation and migration capacities of CACs were impaired in subjects with atherosclerotic risk factors compared with those of healthy subjects (Figure 4A and B). This finding is not new, but is consistent with the findings of other studies. It was not our goal in the present study to elucidate mechanisms responsible for the impaired neovascularization-related capacities of CACs obtained from patients with atherosclerotic risk factors.

5. Conclusions

In vitro LIPUS of CAC cultures was effective to augment neovascularization-related capacities not of only H-CACs but also of P-CACs. LIPUS-pre-treated P-CACs restored the impaired neovascularization capacity of P-CACs in the mouse model of hindlimb ischemia. Thus, LIPUS-pre-treatment of P-CACs may be a new strategy to enhance the clinical effects of therapeutic angiogenesis for critical limb ischemia.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

Acknowledgements

We thank Kimiko Kimura, Miho Kogure, and Miyuki Nishigata from the Cardiovascular Research Institute, Kurume University, for their excellent technical support in the flow cytometry, western blotting, and tissue staining.

Conflict of interest: none declared.

Funding

This study was supported by a Grant-in-Aid from the Strategic Research Foundation for Private Universities from MEXT, Japan, to Kurume University; and another grant from the Japan Cardiovascular Research Foundation to K.S.

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