Treatment of Ischemic Limbs Based on Local Recruitment of Vascular Endothelial Growth Factor-Producing Inflammatory Cells With Ultrasonic Microbubble Destruction

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OBJECTIVES
We sought to clarify the mechanism for neovascularization by ultrasonic microbubble destruction (US/MB) and its ability to improve the function of ischemic limbs.

BACKGROUND
In tissue, US/MB can cause capillary rupture, leading to angiogenesis and arteriogenesis.

METHODS
Seven days after removal of the femoral artery (day 0) in mice, microbubble/ultrasound treatment was performed by intermittent insonation (1.6 MHz, mechanical index 1.1) to the ischemic limbs after intravenous infusion of phospholipid-stabilized microbubbles BR14 (US/MB group). Effects were compared with those in untreated mice with ischemic limbs (control group).

RESULTS
Immunostaining of the treated muscles revealed a greater leukocyte (CD45-positive cell) count in the US/MB group on days 3 and 7. These cells included F4/80-positive cells (macrophages) and CD3-positive cells (T-lymphocytes), both of which were immunoreactive to vascular endothelial growth factor (VEGF) antibody. Muscular VEGF content by Western blotting was elevated in the US/MB group on day 3, which declined but remained greater until day 21. The US/MB group showed a greater capillary density by alkaline phosphatase stain on day 7 without further increase at day 21. Surface vascularity of the muscles and blood flow were greater in the US/MB group on day 7, which further increased by day 21. Moreover, the US/MB group showed a two-fold longer treadmill time compared with the untreated control group on day 21. None of these favorable effects were observed in mice treated with ultrasound only or microbubbles only.

CONCLUSIONS
Ultrasound destruction of microbubbles delivered to the ischemic limbs can recruit inflammatory cells producing VEGF, which is followed by neovascularization and functional improvement, and thus has a therapeutic potential.

Therapeutic neovascularization in ischemic tissues has been stimulated by the targeted delivery of vascular growth factor (VGF) genes (1) and proteins (2). However, a considerable angiogenic effect of the “control” adenoviral agent, which was directly injected into the skeletal muscle, was reported (3). Acupuncture to the myocardium was also reported to induce angiogenesis (4). Transmyocardial laser revascularization therapy elicits neovascularization, which is hypothesized to follow an inflammatory response (5). Therefore, induction of inflammation by local injury attributable to direct injection itself or to proinflammatory stimuli may cause neovascularization. Moreover, although bone marrow-derived mononuclear cells have been transplanted into the ischemic tissue for neovascularization as the source of endothelial progenitor cells, a significant role for inflammatory cells that also differentiate from this cell fraction and produce VGFs has also been noted (6). Thus, inflammatory cells producing VGFs may play key roles in therapeutic angiogenesis.

Because microbubble destruction in tissue microcirculation by ultrasound can cause capillary rupture, we (7,8) and other investigators (9,10) have used this phenomenon as a microinjection in enhancing protein or gene delivery to target organs. Recently, microbubble destruction itself was found to stimulate angiogenesis and arteriogenesis in arterially occluded (11) as well as normal (12) skeletal muscles in rats, resulting in restoration of blood flow during adenosine infusion. Although it was hypothesized that recruitment of inflammatory cells might follow capillary rupture to play a role in this phenomenon, no direct evidence has been provided. Moreover, although it was shown that this phenomenon restored blood flow distal to the occluded artery during pharmacologic vasodilation, its therapeutic potential for limb ischemia limiting exercise capacity remains to be tested.

The purposes of this study were to examine whether ultrasonic microbubble destruction (US/MB) can enhance vascularity in the ischemic skeletal muscle, and if so, to
investigate the mechanism for this phenomenon, especially regarding the role of local inflammatory cell infiltration, and to examine whether the neovascularization thus induced provides functional improvement of the diseased limbs.

METHODS

Mouse model of limb ischemia. The study protocols were approved by the Institutional Animal Care and Use Committee of Kagawa University School of Medicine. In wild-type mice (C57BL/6, Charles River Japan, Yokohama, Japan), the entire right femoral artery and vein were surgically excised under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally) to produce hind limb ischemia (13).

Treatments with ultrasound and/or microbubbles. Seven days after surgery (day 0), the mice were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital and the right jugular vein was cannulated. A saline solution of 2 to 5 \( \times \) 10^8 microbubbles/ml (14) was slowly injected at 2.4 ml/h for 3 min so that approximately 2.4 to 6.0 \( \times \) 10^8 microbubbles were administered. The hind limb to be treated was placed in a custom-designed acoustic coupler for ultrasound exposure. The insonation was performed with an S3 transducer of a SONOS 5500 (Philips Medical Systems, Andover, Massachusetts) in five consecutive transverse planes from proximal to distal levels of the hind limb, leaving no unsonified segments between the planes. We used the harmonic power Doppler mode at 1.6-MHz transmission frequency with a mechanical index of 1.1 to obtain the short-axis image of the limb in the center of the imaging plane, where the acoustic focus was placed (Fig. 1). We suspended the insonation during the injection of BR14 until it was started at the most proximal level with a burst of 20 frames in the multi-frame trigger mode at every 99th cardiac cycle. Then the transducer was serially moved to the next segment for the same insonation procedure until the most distal segment (the fifth segment) was insonated. The series of proximal to distal scans was repeated five times for sufficient destruction of re-circulating microbubbles. During the treatment, microbubble delivery to the ischemic limbs was confirmed by the apparent opacification of the first frame of each burst. In the second group, insonation was performed in the same manner without pre-infusion of microbubbles (US group). The third group received microbubble infusion that was not followed by insonation (MB group). In the control group, no insonation or infusion of microbubbles was performed. Then, subgroups of mice in each group were euthanized 3, 7, or 21 days after treatments to assess capillary density, inflammatory cell infiltration, vascular endothelial growth factor (VEGF) expression, collateral vessel formation (angiogenesis), or blood flow. Ten to 18 mice were euthanized on day 0 (before treatment) to obtain baseline data for each assessment. Six to 12 mice in each group were subjected to serial treadmill tests performed immediately before, 7 days after, and 21 days after treatment.

Histologic assessments. Skeletal muscles harvested from the treated limbs after euthanasia were either fixed in neutral 10% formalin and embedded in paraffin or frozen in Tissue-Tek OCT compound (Sakura Finetek U.S.A., Inc., Torrance, California) and stored at −80°C until sectioning for histologic assessments.

Capillary density. Capillary density within the ischemic thigh adductor muscles was analyzed to evaluate vascularity at the level of microcirculation. Three pieces of the ischemic muscles harvested from each animal were embedded in OCT compound. Tissue sections (5 \( \mu \)m in thickness) were cut from each specimen on a cryostat so that the muscle fibers were oriented in a transverse fashion, and then were placed on glass slides. Sections were stained for alkaline phosphatase to detect capillary endothelial cells (8), and were then counterstained with eosin. Three random microscopic fields at \( \times \)400 magnification from each slice were examined to evaluate capillary density as the number of capillaries per high-power field (15).

Local inflammatory response. One tissue slice adjacent to the one for alkaline phosphatase stain was subjected to immunohistochemical staining using an anti-mouse monoclonal antibody (mAb) to a common leukocyte antigen CD45 (PharMingen, San Diego, California) (15). Briefly, sections were fixed with acetone and were incubated with the primary antibody and thereafter with horseradish peroxidase-conjugated antibody. Sections were colorized with diaminobenzidine and were counterstained with hematoxylin. Three random microscopic fields from each slide...
were examined to count the number of CD45-positive cells per high-power field.

**VEGF expression in inflammatory mononuclear cells.** We examined VEGF expression in mononuclear cells in treated limbs using a double immunofluorescence staining technique (15). Cryostat sections, 5 μm in thickness, from ischemic tissues were mounted on silicone-coated slides. They were then incubated overnight at 4°C with goat anti-mouse VEGF mAb (Santa Cruz Biotechnology, Santa Cruz, California) and either of a marker of macrophages, rat anti-mouse F4/80 mAb (Dainippon Pharmaceutical, Osaka, Japan) or that of T-lymphocytes, rat anti-mouse CD3 mAb (CALTAG Laboratories, Burlingame, California). The slides were then incubated for 30 min at 37°C with a fluorescein isothiocyanate-conjugated anti-goat immunoglobulin G secondary antibody (Vector Laboratories, Burlingame, California) to detect VEGF. Then they were further incubated for 30 min at 37°C with Texas red isothiocyanate-conjugated anti-rat immunoglobulin G (P.A.R.L.S., Compiegne, France) to detect macrophages or T-lymphocytes. The slides were examined and photographed using a laser confocal microscope LSM-G200 (Olympus, Tokyo, Japan) using optimal combinations of specific excitation and emission filters for co-localization analysis by dual fluorescence staining.

**Western blot analysis.** Protein extracts were obtained from homogenized ischemic skeletal muscles. Twenty micrograms of protein per sample was separated on a 12.5% polyacrylamide gel and electroblotted onto polyvinylidene fluoride membranes (Amersham Biosciences, Piscataway, New Jersey). The membrane was blocked with 10% nonfat dry milk in phosphate-buffered saline with 0.2% Tween (T-PBS) and then probed with a goat polyclonal antimouse VEGF antibody (Santa Cruz Biotechnology) for 3 h at room temperature. After incubation with the primary antibody, the membrane was washed in T-PBS and then incubated for 1 h with anti-goat immunoglobulin G conjugated with horseradish peroxidase. The membrane was then washed in T-PBS, and antigen-antibody complexes were visualized using an enhanced chemiluminescence kit (PerkinElmer, Boston, Massachusetts), followed by exposure to chemiluminescence films (Hyperfilm, Amersham Biosciences) (15).

**Analysis of surface vascularity of skeletal muscles.** The skeletal muscles were exposed through the skin incision from the thigh to the ankle. Then, the surface vasculature was observed through the fascia by an intravital microscope (stereomicroscope Leica MZ6, Leica Microsystems, Tokyo, Japan) at ×32 magnification and was digitally recorded. We quantitated the vascularity by the grid method (16). In brief, visible vessels were manually traced on the printout of the magnified digital images, allowing minimal diameter of traceable vessels of approximately 0.01 mm. Subsequently, a virtual grid (equivalent to 0.3 × 0.3 mm in tissue) was superimposed on the printout with the trace lines of vessels at an angle of approximately 45° to the long axis of the limb. Then, we counted the intersections of the trace lines and grid lines for the entire length of the limb to quantitate the vascularity as the number of intersections per square centimeter of surface area of the limb.

**Microsphere analysis.** Blood flow to the ischemic muscles was assessed with the microsphere technique (11,17). In brief, red fluorescent microspheres (Fluo Spheres, 15 μm, Molecular Probes, Inc., Eugene, Oregon) were injected into the left ventricle (150,000 particles) for 1 min before euthanasia. The adductor muscles in the treated ischemic and control non-ischemic limbs were then dissected and frozen. Microspheres were visually counted in the 70-μm cross-sectional slices with a fluorescence microscope (BX-51, Olympus) at ×100 magnification to assess the blood flow in the treated limbs as the percent of control non-ischemic limbs.

**Treadmill walk test.** To quantitate the total leg function, we serially performed treadmill exercise testing using the Rota Rod Treadmill 7600 for mice (Ugo Basile, Varese, Italy). Mice were placed on the horizontal rotating rod (3-cm diameter, 24 rpm). Then the duration on the rod was measured, which was repeated after a 30-min interval for a complete recovery, and the average of the two measurements was used.

**Statistics.** All values are presented as the mean ± standard error of the mean. Analysis of variance followed by the post-hoc Student-Newman-Keuls test was used in all pairwise comparisons (six comparisons) among the four groups. A value of p < 0.05 was considered to be statistically significant. The statistical package SigmaStat, version 2.03 (Jandel Scientific, San Rafael, California), was used for statistical analyses.

**RESULTS**

Twenty of 249 mice died before treatment, during either surgery or cannulation. Forty mice were used for baseline assessments, and the remaining 189 mice were subjected to one of the four treatments. No mice died during treatment or follow-up, until they were euthanized for the scheduled evaluations.

**Inflammatory cell infiltration.** Figure 2 compares serial changes in leukocyte infiltration. The CD45-positive cell count gradually decreased in the control, US, and MB groups; however, the US/MB group showed a three-fold higher cell count than the other three groups three days after treatment (Fig. 2A), which persisted at day 7 (Fig. 2B).  

**Production of VEGF by infiltrating cells.** Figure 3 shows the results of double immunofluorescence staining of macrophages and VEGF (Fig. 3, top) and that of T-lymphocytes and VEGF (Fig. 3, bottom) obtained from the US/MB group on day 3. Distribution of the infiltrated cells agreed well with that of VEGF for both cell types, suggesting that the infiltrated leukocytes included macrophages and T-lymphocytes and that they were the sources of VEGF.
Tissue VEGF concentration. Representative Western blot photographs comparing the control and US/MB group are shown in Figure 4. The VEGF concentration in the treated skeletal muscle decreased in the untreated control group but was increased at day 3 in the US/MB group, resulting in higher expression throughout the observation compared with control (panel A). Although VEGF concentration was similarly reduced in the US group and the MB group, the US/MB group showed a marked elevation of VEGF concentration three days after treatment, which remained higher than the other groups until day 21 (panel B).

Capillary density. Figure 5 shows capillary angiogenesis. A marked increase in capillaries was noted at day 7 only in the US/MB group (panel A). Capillary density was higher, by about 30%, in the US/MB group than in the other groups at day 7. Although no further increase at day 21 was observed, the capillary density remained higher in the US/MB group than in the other groups (panel B).

Surface vascularity. Panel A in Figure 6 shows photographs of vasculature on the surface of the skeletal muscles from an untreated mouse (left) and that in the US/MB group (right) obtained 21 days after treatment. A marked increase in vascularity in the US/MB mice compared with controls was apparent. The vascularity was 1.5-fold higher in the US/MB mice compared with the other groups at day 7, which was further increased at 21 days after treatment (panel B).

Blood flow. Figure 7 compares alterations in blood flow in the treated ischemic limbs. Although only a small increase in blood flow was observed in the control, US, and MB groups, the US/MB group showed significantly greater improvement of blood flow, which was 71% of non-ischemic on day 7 and 89% on day 21.

Treadmill time. Figure 8 compares treadmill time, which tended to increase after treatment in all groups. However,
the increase was greater in the US/MB group, which was approximately two-fold longer than in the other three groups at 21 days after treatment.

**DISCUSSION**

The major findings of the present study are: 1) microbubble destruction by ultrasound can cause angiogenesis and can increase vascularity and blood flow in the ischemic limbs; 2) this phenomenon is associated with the recruitment of inflammatory cells that produce VEGF; and 3) the neovascularization thus produced parallels the improvement of treadmill time.

**Delivery of microbubbles to ischemic tissue.** Angiogenesis by ultrasonic destruction of microbubbles was shown in non-ischemic (12) or collateral-perfused skeletal muscles (11). We have shown in the present study that this method can be applied to ischemic limbs in which resting blood flow is limited, (15) and therefore the delivery of microbubbles with the same rheology as erythrocytes possibly would be limited. The model used in the present study showed spontaneous recovery of blood flow to approximately 50% of that of intact muscles seven days after the removal of the artery (15) when we performed US/MB treatment. In addition to the partial recovery of flow, the specific rheologic characteristics of this agent might play an important role in achieving a sufficient concentration of microbubbles in the target region. A fraction of this agent shows transient retention in the microcirculation after intravenous injection.

**Figure 5.** (A) Alkaline phosphatase stain showing a greater capillary density in US/MB than the other groups on day 21. Bars indicate 50 μm. (B) Such an effect was statistically significant on days 7 and 21. (n = 12 for baseline, three mice were used for each subsequent column.) *p < 0.001 vs. the other groups. Abbreviations as in Figure 2.

**Figure 6.** (A) Photographs showing greater vascularity of treated skeletal muscle in the US/MB group compared with the control group on day 21. Bars indicate 1 mm. (B) Vascularity quantified with the grid method was greater in the US/MB group than in the other groups on days 7 and 21. (n = 5 to 10 for each column.) *p < 0.001 vs. the other groups. Abbreviations as in Figure 2.

**Figure 7.** Augmented restoration of blood flow to the treated ischemic limbs in the US/MB group. (n = 12 for baseline, three mice were used for each subsequent column.) *p < 0.05 vs. MB. †p < 0.05 vs. the other groups. Abbreviations as in Figure 2.

**Figure 8.** Progressive prolongation of treadmill time in the US/MB group. (n = 6 to 12 for each group.) *p < 0.05 vs. the other groups. Abbreviations as in Figure 2.
and contributes to persistent myocardial opacification (14), presumably because of the net negative surface charge providing complement-mediated attachment to the endothelium (18). A three-minute withholding time of ultrasound exposure before the first series of insonation allowed accumulation of microbubbles to a sufficient concentration in the target ischemic planes in our experiment. In fact, we observed a dense opacification of the skeletal muscles on the first frames of the 20 frame bursts during the first series of insonation. Substantial opacification was also obtained during the second series and thereafter with decreasing intensities. Because we insonated five different planes with \(20\) s intervals (99 cardiac cycles) in a series, the interval between insonation at a target transverse plane was: \(20 \times 5 = 100\) s. This long interval between bursts allowed a sufficient concentration of microbubbles in the plane because of their transient retention in the microcirculation (14). In addition, because the opacification of the skeletal muscles rapidly decreased during a burst, it is speculated that microbubbles were sufficiently disrupted by the ultrasound energy and conditions we used in the present study.

**Mechanisms for angiogenesis and arteriogenesis.** The exact mechanism for these phenomena has not been elucidated in previous studies, although inflammation after injury was speculated to contribute. Therefore, we examined the role of inflammatory cells in the angiogenesis especially as the source of VEGF. We have directly shown that inflammatory cell infiltration was augmented after the injury created by US/MB. The US/MB-recruited inflammatory cells including macrophages and T-lymphocytes expressed VEGF, which was associated with the increase in capillary density and vascularity and resting blood flow. Thus, the local increase in concentration of VGFs secreted by infiltrated inflammatory cells is likely to be the mechanism for these phenomena. However, further mechanism(s) for the induction of inflammatory cell infiltration by US/MB remain(s) uncertain, although it was postulated that capillary rupture caused inflammation (14). The shock wave and cavitation produced by US/MB causes capillary rupture that changes shear stress to the endothelium and increases oxidative stress to the surrounding cells, which may initiate the inflammatory process. Our previous studies have shown that this agent can be targeted to infiltrated macrophages (19,20). Therefore, it is possible that microbubbles were delivered to the infiltrated leukocytes in the ischemic region and disrupted them at exposure to the ultrasound, and thereby caused leakage of proinflammatory chemokines from these cells (19).

Although the number of capillaries was already greater in the US/MB group 7 days after treatment, no further increase in capillary density was noted at day 21. In contrast, vascularity with a caliber size more than 0.01 mm increased from day 7 to day 21, which paralleled the improvement of blood flow and treadmill time. Therefore, angiogenesis may not directly contribute to the improvement of limb function but preceded the increase in vascularity due to formation and increase in caliber of arterioles. Angiogenic factors released from inflammatory mononuclear cells include basic fibroblast growth factor (21) that activates several cell types in the vascular wall (22) and dilates pre-existing vessels, referred to as arteriogenesis, rather than the new growth of capillaries to which VEGF contributes (23). Although we did not show the augmentation of local expression of growth factors other than VEGF, it is likely that expression of other factors was also enhanced either directly or indirectly by US/MB, which resulted in a delayed increase in blood vessels larger than capillaries.

**Clinical implications.** An increase in vascularity produced by US/MB could be a mere unorganized and useless post-inflammatory process, whereas both capillary rupture and subsequent inflammation could be harmful to the tissue, canceling the benefit of neovascularization. Therefore, we evaluated the function of treated limbs as determined by exercise capacity by using the treadmill test used in previous clinical studies on therapeutic angiogenesis for arterial occlusive disease in lower limbs (1). Recovery of treadmill time was significantly augmented by the US/MB treatment, which resulted in a two-fold increase on day 21. Thus, the post-inflammatory angiogenesis produced by US/MB is valuable, although possible neuroablation by US/MB could also contribute to the improved treadmill time by reducing pain perception. Therefore, although creation of injury to the ischemic skeletal muscles is requisite, the net outcome of this method is profitable. In addition, this method can eliminate the potential risk of local infection in the ischemic region caused by trans-cutaneous injection. Moreover, the noninvasiveness of this method also allows repeated treatment to potentially maximize the effects.

**Study limitations.** There are several limitations to this study. We tested the method only in small animals, which provided an ideal condition for insonation. For the application to patients, the insonation condition needs to be optimized to obtain the best improvement of leg function, which may include the tradeoff between the deterioration directly caused by the acoustic injury and the improvement by angiogenesis after the injury. Future studies to optimize the bioeffects should include ultrasound dosimetry, optimization of frequency, pulse cycle length, and acoustic intensity, as well as identification or fabrication of effective microbubbles and their doses.

Second, although the net outcome of this method was beneficial, none of the possible adverse effects, including pain, fever, and systemic inflammatory response, were addressed.

Third, the model we used is for acute limb ischemia that shows spontaneous angiogenesis without treatments (15). Therefore, it remains to be clarified whether this method may be applicable to chronic stable ischemia or to only slightly ischemic or normal tissues showing little spontaneous angiogenesis. Possible options for enhancing the efficacy in such settings may include systemic co-administration of agents that potentially augment local inflammatory cell
infiltration, such as monocyte chemoattractant protein-1 (24) and granulocyte-colony-stimulating factor (25). Possible advantageous effects of the repeated application of this minimally invasive method may also be worth testing in future studies. This may be especially beneficial in the case of severe ischemia that limits microbubble supply to the central zone of ischemic regions. Microbubbles delivered to the marginal zone of the ischemic area can produce neovascularization that provides flow for the microbubble delivery in the subsequent session.

Finally, effects of other agents clinically used in treating obstructive arterial disease, including angiotensin II type-1 receptor antagonists that exert anti-inflammatory actions (15), remain to be examined in the future.

CONCLUSIONS

Recruitment of inflammatory cells in ischemic skeletal muscle in mice by ultrasonic microbubble destruction can induce angiogenesis resulting in increased vascularity and blood flow, which leads to functional improvement. Our data may provide a basis for developing a safe and useful tool for therapeutic angiogenesis.

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