

● *Original Contribution*

GRANULOCYTE COLONY-STIMULATING FACTOR FACILITATES THE ANGIOGENESIS INDUCED BY ULTRASONIC MICROBUBBLE DESTRUCTION

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Abstract—Ultrasonic destruction of microbubbles (US/MB) in the microcirculation causes local inflammatory cell infiltration, which has been shown to induce angiogenesis. Granulocyte colony-stimulating factor (G-CSF), which mobilizes myelomonocytic cells from the bone marrow and enhances vascular endothelial growth factor (VEGF) release from these cells, has also been applied to therapeutic angiogenesis induction. In the present study, we sought to examine the potential of G-CSF pretreatment to enhance the angiogenic effect of US/MB. Ischemic hindlimbs in mice were treated with either a predetermined minimal effective dose (300 $\mu\text{g}/\text{kg}$) of G-CSF, US/MB alone or G-CSF pretreatment followed by US/MB at seven days after removal of the femoral artery. Ultrasonic destruction of microbubbles was performed as intermittent pulsed local insonation using a diagnostic ultrasound scanner at a peak negative pressure of 1.4 MPa after intravenous injection of perfluorocarbon microbubbles. At 21 days after the treatment, we quantified the surface vascularity using a grid method and the capillary density using an alkaline phosphatase stain. Relative to the capillary density in normal muscle, the capillary density in the treated limbs was restored to $74 \pm 13\%$ by G-CSF alone and $90 \pm 20\%$ by US/MB alone ($p < 0.05$ vs. both untreated and G-CSF alone), and further increased to $101 \pm 21\%$ by G-CSF pretreatment. The collateral growth induced by the combination of G-CSF pretreatment and US/MB was 2.8- and 1.4-fold greater than the growth induced by G-CSF alone and US/MB alone, respectively ($p < 0.05$ for both). Thus, pretreatment with a single minimal effective dose of G-CSF can augment the angiogenic effect of US/MB. (E-mail: komori@med.kagawa-u.ac.jp) © 2007 World Federation for Ultrasound in Medicine & Biology.

Key Words: Capillary density, Collateral vessel, Hindlimb ischemia model.

INTRODUCTION

Ultrasonic destruction of microbubbles (US/MB) in the microcirculation can cause capillary rupture in target tissues (Li et al. 2003a; Miller et al. 2000; Skyba et al. 1998). This phenomenon has been applied not only to the local delivery of bioactive agents (Chen et al. 2003; Kondo et al. 2004; Li et al. 2003b; Shohet et al. 2000; Takeuchi et al., 2003), but also to the induction of angiogenesis or microvascular remodeling (Song et al. 2002, 2004). Using a mouse model of hindlimb ischemia, we have shown that application of US/MB to ischemic limbs can induce angiogenesis that results in functional

improvement of the treated limbs, thereby suggesting its potential for safe therapeutic angiogenesis induction (Yoshida et al. 2005). The angiogenesis induced by US/MB was found to be caused by enhanced local infiltration of inflammatory cells releasing vascular endothelial growth factor (VEGF) in response to microinjuries or microhemorrhages (Yoshida et al. 2005).

Granulocyte colony-stimulating factor (G-CSF) has also been applied to therapeutic angiogenesis induction in animal models (Hasegawa et al. 2006; Jeon et al. 2006; Lee et al. 2005; Ohki et al. 2005; Takagi et al. 2005) and humans (Deindl et al. 2006; Kang et al. 2004; Ripa et al. 2006; Suzuki et al. 2006; Wang et al. 2005; Zohlhofer et al. 2006). Although various devices have been developed to increase the efficacy of angiogenesis in these studies, optimal methods using systemic administration of G-CSF have not yet been established. The mechanisms for the angiogenic effects of G-CSF are

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partly attributable to increased numbers of myelomonocytic cells in the circulation and their release of VEGF, in addition to the mobilization of endothelial progenitor cells (Ohki *et al.* 2005). Considering the sequence of the mechanisms of angiogenesis induction by G-CSF and US/MB, we hypothesized that pretreatment with G-CSF would augment the angiogenesis induced by US/MB. In the present study, we sought to examine the potential of combining G-CSF preadministration with US/MB for therapeutic angiogenesis induction.

MATERIALS AND METHODS

Mouse model of limb ischemia

The study protocols were approved by the Institutional Animal Care and Use Committee of our institute. In 7-week-old wild-type mice (C57BL/6; Charles River Japan, Yokohama, Japan) weighing 22.5 ± 0.76 g, the entire right femoral artery and vein were surgically excised under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneal [IP]) to produce hindlimb ischemia (Couffinhal *et al.* 1998). The mice were then randomized to various treatments performed at six and seven days after the surgery and maintained until they were euthanized at 28 days after the surgery for evaluation of the treatment effects on the vascularity in their limbs.

Granulocyte colony-stimulating factor treatments

First, we determined the minimal effective dose and frequency of preadministration of G-CSF. At six days after surgery, the mice were treated with a subcutaneous

injection of 0.1 mL of saline or recombinant human G-CSF (rhG-CSF; Kirin Brewery Co. Ltd., Tokyo, Japan) solution at 100, 200, 300 or 400 $\mu\text{g}/\text{kg}$ body weight ($n = 6-8$ per group). In another group of mice ($n = 6$), subcutaneous injections of G-CSF at 100 $\mu\text{g}/\text{kg}/\text{day}$ were repeated at 3, 4, 5 and 6 days after removal of the femoral artery.

Ultrasound and microbubble treatments

At seven days after surgery, the mice were anesthetized with an IP injection of pentobarbital (50 mg/kg) and the right jugular vein was cannulated. Ultrasound destruction of microbubbles was performed as described previously (Yoshida *et al.* 2005). The hindlimb to be treated was placed in an acoustic coupler made of degassed agar on a cork board such that cross sections of the limb were exposed to ultrasound. We used the S3 diagnostic ultrasound transducer of a SONOS5500 system (Philips Medical Systems, Andover, MA, USA) in the power Doppler mode to obtain a short-axis color image of the limb in the center of the imaging plane at a depth of 3 cm (Fig. 1a). This mode used different ultrasound pulses for color imaging (transmission frequency: 3.2 MHz; mechanical index: 0.8) and gray level imaging (transmission frequency: 1.6 MHz; mechanical index: 1.1) at a pulse repetition frequency of 2.5 kHz. For sufficient microbubble destruction, we used the "multi-frame trigger mode" in which bursts of ultrasound pulses for a period of 20 frames were emitted at the end-diastole of each cardiac cycle as described later. Regarding these

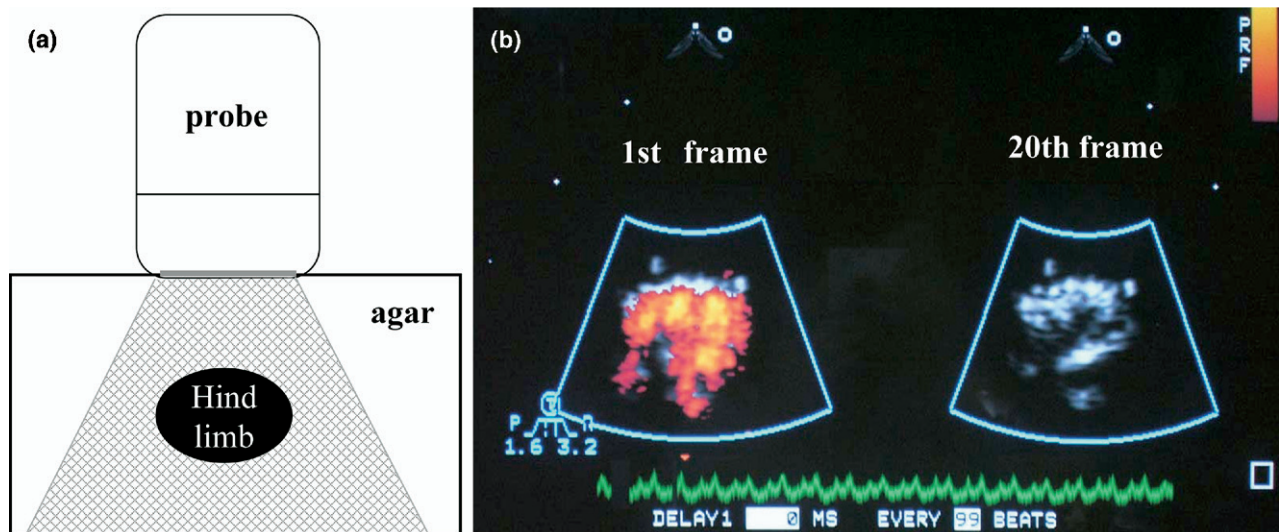


Fig. 1. Microbubble destruction by ultrasound in the ischemic limbs. The ischemic limb was placed in an acoustic coupler. Ultrasound was applied to obtain a short axis image as shown in the right panels. Opacification is apparent on the first frame and is absent in the 20th frame, indicating both sufficient microbubble delivery to the ischemic limbs and complete microbubble destruction by a 20-frame burst. The cross-hatched area denotes the field insonated by the power Doppler mode.

settings, ultrasound pulses with a peak negative pressure of 1.4 MPa were applied to the target and estimated to result in a spatial peak temporal average of acoustic intensity of 15 mW/cm^2 during the 20-frame burst, assuming that the attenuation from passage through the tissue was 0.3 dB/cm.

While insonation was suspended, a saline solution of microbubbles (BR14; Bracco SA, Geneva, Switzerland) at a concentration of 2 to 5×10^8 microbubbles/mL (Fisher et al. 2002) was injected at 2.4 mL/h for 3 min, such that approximately 2.4 to 6.0×10^7 microbubbles were administered without exposure to ultrasound. On completion of the microbubble injection, insonation was started at the most proximal level of five consecutive transverse planes from the proximal to distal levels of the hindlimb. A burst of 20 frames in the multiframe trigger mode with a burst interval of 99 cardiac cycles was applied to the segment. The transducer was then moved to the next segment to be subjected to the second burst, and this was serially performed until the most distal segment (fifth segment) of the limb was insonated, leaving no uninsonated segments between the planes. The series of proximal to distal scans were repeated five times for sufficient destruction of any recirculating microbubbles. During the treatment, microbubble delivery to the ischemic limbs was confirmed by the apparent opacification of the first frame of each burst (Fig. 1b).

Study groups

We compared four treatment groups ($n = 9$ – 10 per group). In the untreated group, saline was administered instead of G-CSF at six days after the induction of ischemia and saline was injected instead of microbubbles on the next day, and this was not followed by insonation. In the G300 group, a subcutaneous injection of G-CSF at $300 \mu\text{g/kg}$ was given at six days after the surgery and saline was injected from the jugular vein on the next day, and this was not followed by insonation. The

US/MB group was injected with saline at six days after the surgery and subjected to the US/MB treatment on the next day. Finally, the G+US/MB group was pretreated with a subcutaneous injection of G-CSF at 6 days after the surgery and received the US/MB treatment on the next day. The treatment protocols are summarized in Fig. 2.

Analysis of the surface vascularity of skeletal muscles

Before the mice were euthanized at 28 days after surgery, their skeletal muscles were exposed from the thigh to the ankle through a skin incision. The surface vasculature was observed through the fascia using an intravital microscope (Stereomicroscope Leica MZ6; Leica Microsystems, Tokyo, Japan) at $32\times$ magnification and digitally recorded. We quantified the vascularity using a previously described grid method (Parsons-Wingerter et al. 1998; Yoshida et al. 2005). Briefly, the identification of each image was blinded, and visible vessels were manually traced on a printout of the magnified digital image, with a minimal diameter of traceable vessels of approximately 0.1 mm. Subsequently, a virtual grid (equivalent to $0.3 \times 0.3 \text{ mm}$ in the tissue) was superimposed on the printout with the trace lines of the vessels at an angle of approximately 45 degrees to the long axis of the limb (Fig. 3a). The intersections of the trace lines and grid lines were counted for the entire length of the limb to quantify the vascularity as the number of intersections/ cm^2 surface area of the limb. When counting the intersections in normal limbs from normal mice, the saphenous artery and vein were excluded and only distal vessels were included to derive values comparable to those in the other groups in which these main vessels were surgically removed. Collateral growth was defined as an increase in vascularity above the average of that obtained in normal limbs harvested from normal untreated mice at the same age (11 weeks; $n = 5$).

Capillary density

After the intravital microscopic observations, the mice were euthanized at 28 days after surgery. Skeletal muscles harvested from the treated limbs were frozen in Tissue-Tek OCT compound (Sakura Finetek U.S.A., Inc., Torrance, CA, USA) and stored at -80°C until sectioning for histologic assessment. The capillary density within the ischemic thigh adductor muscles was analyzed. Tissue sections ($5 \mu\text{m}$ in thickness) were cut from each specimen on a cryostat, such that the muscle fibers were oriented in a transverse fashion, and then placed on glass slides. The sections were stained for alkaline phosphatase to detect capillary endothelial cells (Takagi et al. 2005; Yoshida et al. 2005). Figure 3b shows representative photomicrographs at $400\times$ magni-

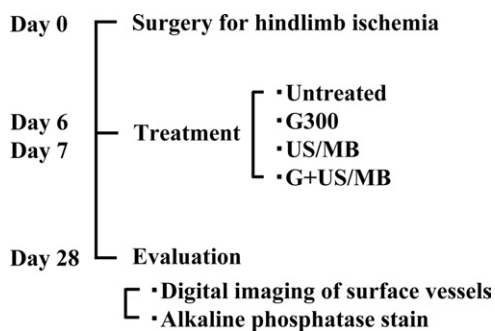


Fig. 2. Treatment protocols. G300: pretreatment with $300 \mu\text{g/kg}$ G-CSF alone; US/MB = ultrasonic microbubble destruction alone; G+US/MB = preadministration of G-CSF ($300 \mu\text{g/kg}$) followed by US/MB.

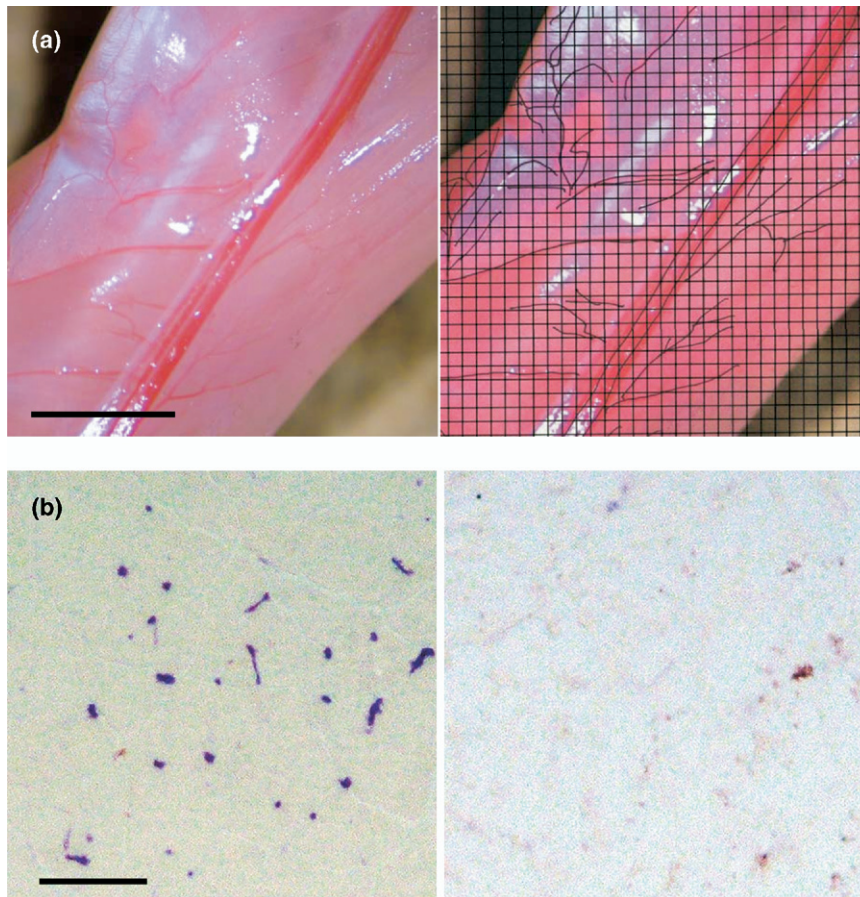


Fig. 3. Intravital microscopic observation of surface vascularity of a treated limb (a–left) and its quantification by the grid method (a–right). After alkaline phosphatase staining, the capillaries are observed as purple dots surrounding the cross sections of muscle fibers in a normal limb (b–left). A few of these capillaries are observed in avascular or hypovascular adipose tissue obtained from a fat layer as a negative staining control (b–right). The scale bar in (a) is 3 mm, and that in (b) is 100 μm .

fication of a normal limb (left) and avascular or hypovascular adipose tissue from a fat layer as a negative staining control (right). The identification of the specimens was blinded, and three random microscopic fields at 400 \times magnification were examined in each slice to evaluate the capillary density as the number of capillaries per high-power field (HPF) (Sasaki *et al.* 2002; Yoshida *et al.* 2005). Normal untreated mice at the same age (11 weeks) provided the normal value for the capillary density. The recovery of the capillary density after the various treatments was defined as the percentage of the mean value found in normal untreated mice.

Statistics

All values are presented as the mean \pm SD. Analysis of variance was used for comparisons among multiple groups, followed by the *post hoc* Bonferroni's *t*-test (Fisher *et al.* 2002; Kondo *et al.* 2004; Takeuchi *et al.*, 2003) for all pair-wise comparisons. Values of $p < 0.05$ were considered to be statistically significant. A statisti-

cal package (SigmaStat Ver 2.03; Jandel Scientific, San Rafael, CA, USA) was used for all analyses.

RESULTS

Optimal dose and frequency of G-CSF preadministration

Table 1 shows comparisons of the capillary densities and surface vascularity among the ischemic limbs in the groups treated with various doses of G-CSF. Although a single administration of 400 $\mu\text{g}/\text{kg}$ resulted in a greater capillary density compared with those in the untreated, 100- and 200- $\mu\text{g}/\text{kg}$ -treated groups, a single dose of 300 $\mu\text{g}/\text{kg}$ resulted in a similar increase in capillary density to that induced by 400 $\mu\text{g}/\text{kg}$. Repeated administration of 100 $\mu\text{g}/\text{kg}/\text{day}$ (total: 400 $\mu\text{g}/\text{kg}$) failed to increase the capillary density. Similarly, although a single administration of 400 $\mu\text{g}/\text{kg}$ resulted in greater collateral growth compared with those in the untreated, 100- or 200 $\mu\text{g}/\text{kg}$ -treated groups, no statistical difference was obtained relative

Table 1. Effects of various doses of G-CSF on vascularization in the ischemic limbs at 28 days after removal of the femoral artery

| | Capillary density (capillaries/HPF) | Surface vascularity (intersections/cm ²) |
|-----------|-------------------------------------|--|
| Untreated | 31.1 ± 8.6 | 244.0 ± 48.7 |
| G100 | 29.1 ± 6.6 | 245.5 ± 53.7 |
| G200 | 30.5 ± 4.6 | 291.4 ± 37.8 |
| G300 | 44.1 ± 10.7*†‡ | 320.3 ± 47.8* |
| G400 | 49.0 ± 9.4*†‡ | 367.3 ± 29.5*†‡ |
| G100 × 4 | 31.9 ± 4.1§¶ | 323.7 ± 53.8* |

G100, G200, G300 and G400 denote subcutaneous single dose injections of G-CSF at 100, 200, 300 and 400 $\mu\text{g}/\text{kg}$ body weight at 6 days post ischemia. G100 × 4 denotes repeated injections of 100 $\mu\text{g}/\text{kg}$ at 3, 4, 5 and 6 days post ischemia.

* $p < 0.05$ vs. untreated.

† $p < 0.05$ vs. G100.

‡ $p < 0.05$ vs. G200.

§ $p < 0.05$ vs. G300.

¶ $p < 0.05$ vs. G400 by Bonferroni's t -test.

to a single administration of 300 $\mu\text{g}/\text{kg}$. Thus, the minimal dose of G-CSF that significantly augmented the recovery of the vessel numbers was a single administration of 300 $\mu\text{g}/\text{kg}$. Therefore, we chose to use

a single injection of 300 $\mu\text{g}/\text{kg}$ in our subsequent experiments to evaluate the synergistic effect of G-CSF preadministration and US/MB on angiogenesis.

Effect of preadministration of G-CSF on US/MB-induced angiogenesis

Figure 4 shows representative photomicrographs of alkaline phosphatase staining of limbs from the treated groups and a normal mouse. Although the capillary density was greater in the G300 and US/MB groups than in the untreated group, it was further increased in the G+US/MB group to a comparable level to that in the normal limb. Regarding the surface vascularity, Fig. 5 shows representative intravital microscopic photographs of the surface vascularity of limbs from the treated groups and a normal mouse, with the original photographs at the top and those with the trace lines of the vessels at the bottom. As shown on the left, spontaneous collateral growth was noted in the untreated limb compared with the normal limb. Although the G300 and US/MB groups showed increased surface vascularity compared with the untreated group shown on the right,

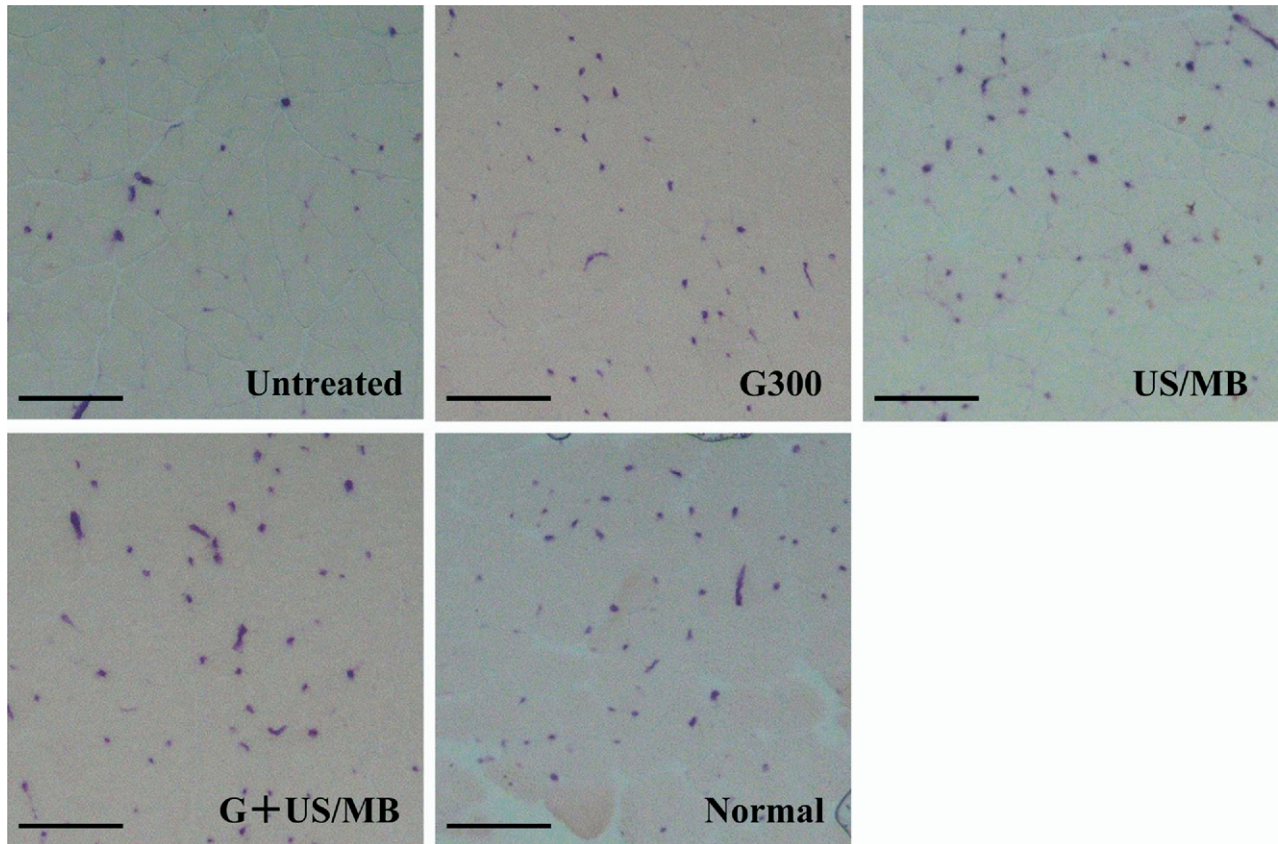


Fig. 4. Representative photomicrographs of alkaline phosphatase staining. Scale bars indicate 100 μm . G300 = pretreatment with 300 $\mu\text{g}/\text{kg}$ G-CSF alone; US/MB = ultrasonic microbubble destruction alone; G+US/MB = preadministration of G-CSF (300 $\mu\text{g}/\text{kg}$) followed by US/MB.

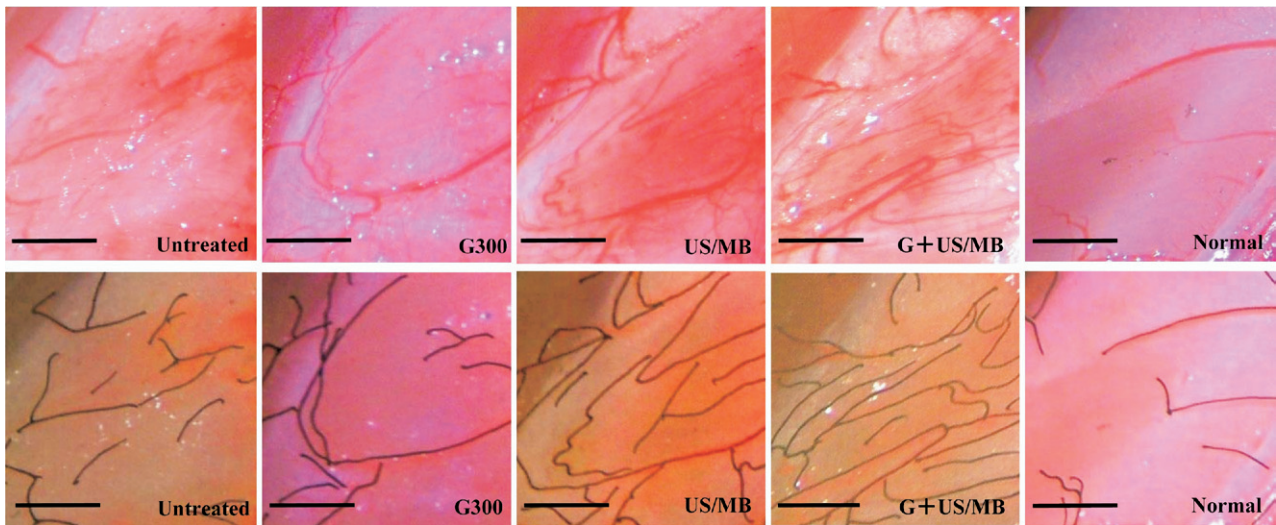


Fig. 5. Representative intravital microphotographs showing the surface vascularity of treated limbs and a normal limb. The original microphotographs (top panels) and those with trace lines of the collateral vessels (bottom panels) are shown. Scale bars indicate 1 mm. G300 = pretreatment with 300 $\mu\text{g}/\text{kg}$ G-CSF alone; US/MB = ultrasonic microbubble destruction alone; G+US/MB = preadministration of G-CSF (300 $\mu\text{g}/\text{kg}$) followed by US/MB.

the G+US/MB group exhibited further augmentation of the collateral growth. The results of these quantitative analyses of the capillary densities and surface vascularity in limbs in the treated groups are summarized in Table 2.

Figure 6 shows a comparison of the extents of recovery of the capillary density in the various treatment groups, defined as the percentage of the average value for normal limbs (56 capillaries/HPF). The G300 group showed a greater capillary density than the untreated group ($p < 0.05$). The US/MB group achieved a substantial, but incomplete, recovery of the capillary density ($90 \pm 20\%$ of the normal control value; $p < 0.05$ vs. both untreated and G300), which was nonsignificantly enhanced by preadministration of G-CSF ($101 \pm 21\%$) to

the level of normal limbs. Thus, preadministration of G-CSF at 300 $\mu\text{g}/\text{kg}$ increased the angiogenesis induced by US/MB alone to the level observed in nonischemic limbs.

Table 2. Effects of various combinations of treatment modes with G-CSF and/or US/MB on vascularization in the ischemic limbs at 28 days after removal of the femoral artery

| | Capillary density (capillaries/HPF) | Surface vascularity (intersections/ cm^2) |
|-----------|-------------------------------------|---|
| Untreated | 32.0 ± 7.9 | 222.7 ± 28.5 |
| G300 | $41.4 \pm 7.0^*$ | $283.0 \pm 51.3^*$ |
| US/MB | $50.4 \pm 11.2^{*\dagger}$ | $360.5 \pm 61.4^{*\dagger}$ |
| G+US/MB | $56.9 \pm 12.0^{*\dagger}$ | $430.4 \pm 36.9^{*\dagger\ddagger}$ |
| Normal | $56.2 \pm 8.8^\dagger$ | $202.4 \pm 22.0^{\dagger\ddagger\§}$ |

G300 = pre-treatment with 300 $\mu\text{g}/\text{kg}$ G-CSF alone; US/MB = ultrasonic microbubble destruction alone; G+US/MB = pre-administration of G-CSF (300 $\mu\text{g}/\text{kg}$) followed by US/MB.

* $p < 0.05$ vs. untreated.

† $p < 0.05$ vs. G300.

‡ $p < 0.05$ vs. US/MB.

§ $p < 0.05$ vs. G+US/MB by Bonferroni's *t*-test.

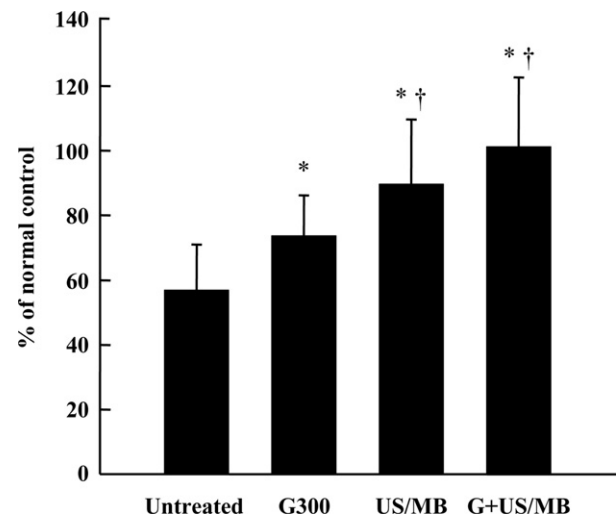


Fig. 6. Recovery of capillary density in the ischemic muscle at 28 days after removal of the femoral artery assessed by alkaline phosphatase staining. Ultrasound destruction of microbubbles restores the capillary density to 90% of the normal level, and the density is further enhanced nonsignificantly by pretreatment with G-CSF to a similar level as that in the normal control limb. * $p < 0.05$ vs. untreated. † $p < 0.05$ vs. G300 by Bonferroni's *t*-test. G300 = pretreatment with 300 $\mu\text{g}/\text{kg}$ G-CSF alone; US/MB = ultrasonic microbubble destruction alone; G+US/MB = preadministration of G-CSF (300 $\mu\text{g}/\text{kg}$) followed by US/MB.

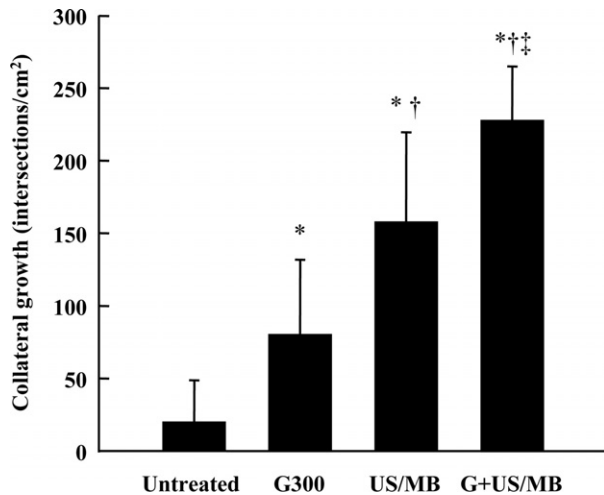


Fig. 7. Extent of collateral vessel growth at 28 days post ischemia. The increases in the numbers of intersections in the grid and the trace lines above the average values for the control limb were compared. Pretreatment with G-CSF further enhances the collateral growth induced by US/MB. * $p < 0.05$ vs. untreated. † $p < 0.05$ vs. G300. ‡ $p < 0.05$ vs. US/MB by Bonferroni's t -test. G300 = pretreatment with 300 $\mu\text{g}/\text{kg}$ G-CSF alone; US/MB = ultrasonic microbubble destruction alone; G+US/MB = preadministration of G-CSF (300 $\mu\text{g}/\text{kg}$) followed by US/MB.

Figure 7 shows a comparison of the collateral growths among the treatment groups, defined as the increase in surface vascularity relative to the average value for normal control limbs (202 intersections/cm²). While the G-300 and US/MB groups showed enhanced collateral growth by 4.0- and 7.9-fold, respectively, relative to the untreated control group (both $p < 0.05$ vs. untreated), the G+US/MB group showed further collateral growth that was 11.4-fold greater than that in the untreated control group ($p < 0.05$ vs. both G300 and US/MB). Thus, preadministration of G-CSF clearly added an arteriogenic effect to the collateral growth produced by US/MB alone.

DISCUSSION

The major finding of the present study is that a single preadministration of G-CSF can complement the angiogenic effect of US/MB alone on ischemic skeletal muscles and restore the capillary density to the normal level. This combined therapy augmented both the collateral growth and angiogenesis, and the effects were significantly greater than those produced by G-CSF alone or US/MB alone. Thus, the present study demonstrated that G-CSF preadministration enhanced the effects of US/MB, thereby increasing therapeutic angiogenesis.

Underlying hypothesis for the additive effect of G-CSF pretreatment and US/MB

Because US/MB in the tissue microcirculation can cause capillary rupture, we (Kondo et al. 2004; Takeuchi et al. 2003) and other investigators (Chen et al. 2003; Li et al. 2003b; Shohet et al. 2000) have used this phenomenon as a means of microinjection for enhancing protein or gene delivery to target tissues. Recently, microbubble destruction itself was found to stimulate angiogenesis and arteriogenesis in arterially occluded muscles (Song et al. 2004), as well as normal skeletal muscles in rats, resulting in a restoration of the blood flow during adenosine infusion (Song et al. 2002). We have shown directly that local inflammatory cell infiltration in ischemic muscles is augmented after capillary rupture by US/MB (Yoshida et al. 2005). Moreover, the leukocytes thus recruited expressed VEGF, which was associated with increases in the capillary density, vascularity and resting blood flow, and it improved limb function (Yoshida et al. 2005).

On the other hand, G-CSF has been used to promote angiogenesis on its own (Ohki et al. 2005) or in combination with other growth factors (Jeon et al. 2006) or bone marrow cell transplantation (Takagi et al. 2005). Recently, it has been shown that, in addition to mobilization of endothelial progenitor cells from the bone marrow, G-CSF increases the number of myelomonocytic cells, including neutrophils, in the circulation and stimulates their release of VEGF, which plays a significant role in the mechanisms of angiogenesis (Ohki et al. 2005). Thus, it is conceivable that the observed synergistic effects of the two treatments would be exerted through mobilization of bone marrow leukocytes in the circulation by G-CSF and stimulation of local leukocyte infiltration by US/MB, as well as stimulation of VEGF production by the infiltrating cells by G-CSF.

Determination of the G-CSF dose for pretreatment

Because there were no existing data for the effects of a single systemic injection of any dose of G-CSF in the mouse hindlimb ischemia model, we tested various incremental doses of G-CSF from 100 to 400 $\mu\text{g}/\text{kg}$. Although administration of the highest dose (400 $\mu\text{g}/\text{kg}$) at six days after removal of the femoral artery resulted in a significantly greater increase in capillary density at 28 days after surgery compared with that in the untreated, 100- or 200- $\mu\text{g}/\text{kg}$ -treated groups, the dose of 300 $\mu\text{g}/\text{kg}$ showed a similar angiogenic effect to that of 400 $\mu\text{g}/\text{kg}$. A total dose of 400 $\mu\text{g}/\text{kg}$ administered at 100 $\mu\text{g}/\text{kg}/\text{day}$ for four consecutive days resulted in no significant increase in the capillary density and a similar increase in vascularity to that produced by the single dose of 300 $\mu\text{g}/\text{kg}$. Therefore, we chose a single injection of 300 $\mu\text{g}/\text{kg}$, which was considered to be the minimal effective

dose and the least likely to show adverse effects, to test the ability of G-CSF preadministration to enhance the angiogenic effect of US/MB.

Angiogenesis and arteriogenesis

In the present study, we confirmed that US/MB substantially increases the vascularity in this ischemia model and newly found that the capillary density recovery was up to $90 \pm 20\%$ of the level in normal skeletal muscle. Although the difference in capillary density between the G+US/MB and US/MB groups did not reach statistical significance, preadministration of G-CSF augmented the increase in capillary density induced by US/MB up to $101 \pm 21\%$ of the level in normal limbs. Thus, the angiogenic effect of US/MB was complemented by preadministration of G-CSF, resulting in a statistically significant increase in collateral growth in the G+US/MB group compared with that in the US/MB group. Therefore, the combination of G-CSF preadministration and US/MB is likely to enhance the process of arteriogenesis or remodeling of latent collaterals (Song *et al.* 2002, 2004), which was shown to follow angiogenesis and be correlated with the extent of improvement of perfusion and function in our previous study (Yoshida *et al.* 2005).

Clinical implications

The effects of G-CSF preadministration on the US/MB-induced recovery of vascularity in ischemic hindlimbs are potentially applicable to the treatment of myocardial ischemia, for which the therapeutic impact of G-CSF has been investigated (Deindl *et al.* 2006; Hasegawa *et al.* 2006; Kang *et al.* 2004; Ripa *et al.* 2006; Suzuki *et al.* 2006; Wang *et al.* 2005; Zohlhofer *et al.* 2006). In recent clinical studies that used G-CSF to treat myocardial infarction, subcutaneous injection of $10 \mu\text{g}/\text{kg}/\text{d}$ for several days resulted in no functional improvement evaluated by left ventricular wall thickening by magnetic resonance imaging (Ripa *et al.* 2006) or no recovery of perfusion assessed by myocardial scintigraphy (Zohlhofer *et al.* 2006). Furthermore, systemic administration at this dose ($10 \mu\text{g}/\text{kg}/\text{d}$ for 4 d) frequently caused in-stent restenosis, presumably as a result of plaque growth or differentiation of the mobilized progenitor cells into smooth muscle cells (Kang *et al.* 2004). Administration of a lower dose ($5 \mu\text{g}/\text{kg}/\text{day}$) for six days resulted in no significant improvement of left ventricular function in patients with myocardial infarction, although no serious adverse effects were seen (Wang *et al.* 2005). Thus, treatment of ischemic myocardium by systemic administration of G-CSF alone seems to be difficult and awaits alternative approaches. In this regard, our method does not involve the full effects of G-CSF, which would require high doses, but uses the minimal

effects of G-CSF, such as mobilization of bone marrow cells to enhance local infiltration of inflammatory cells and local release of VEGF from these cells.

Limitations of this study

There are several limitations of the present study. First, although further increases in the plasma levels and tissue contents of VEGF and inflammatory cells over US/MB alone are likely to be provided by G-CSF pre-treatment, we did not directly show these data. Second, we preadministered G-CSF at one day before US/MB but did not test other pretreatment times. However, because the dose and timing used in our study resulted in almost complete restoration of the capillary density, other modes of preadministration of G-CSF may result in marginal improvement. Third, we did not show direct data regarding functional improvement, such as perfusion and muscle function, in the treated groups. However, we have already shown in our previous study that enhancement of collateral growth is correlated to functional improvement, as assessed by the treadmill walk-time, in this model (Yoshida *et al.* 2005). Fourth, although we estimated the acoustic intensity at the target site, we did not measure the actual value. The acoustic energy delivered to the target would be a function of not only the characteristics of the emitted ultrasound but also its reflections, which were not taken into account and would interfere with or augment the acoustic effects on the target site. Finally, we did not assess the adverse effects of G-CSF alone or in combination with US/MB. Possible hyperneutrophilia or systemic inflammatory responses should be addressed before any clinical applications are proposed.

CONCLUSIONS

In the present study using a mouse hindlimb ischemia model, we have shown that preadministration of a single dose of G-CSF can potentiate the angiogenic effect of US/MB at the target site.

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