

**Figure 3.** Electron-microscopic appearance of endothelium stimulated by Bubble-US+BM-MNCs. Electron-microscopy showing normal endothelium covered with a monolayer coat (Normal) and appearance of Bubble+US+BM-MNCs i.v.-stimulated endothelium. Attachment of both platelets associated with fibrin network and MNCs was observed on all samples treated by Bubble+US+BM-MNCs i.v. Representative figures are shown (n=4 in each group). Bar=10  $\mu$ m.

stimulated by Bubble+US in the medium without platelets. Because we found that adhesion of the fibrin network including platelets was consistently observed on the surface of endothelium in all Bubble+US-treated samples (Figure 3), we next examined the involvement of platelet-derived factors in the adhesion of BM-MNCs. Interestingly, the expression of P-selectin (red fluorescence, right panel in Figure 4) and ICAM-1 was markedly induced in both HUVECs and attaching glycoprotein Ib–positive platelets (merged, yellow), when HUVECs were stimulated by Bubble+US in the medium including platelets (only P-selectin data shown in Figure 4).

We also studied whether factors released from the platelets stimulated by Bubble+US are involved in the induction of adhesion molecules. Therefore, we examined the effect of the

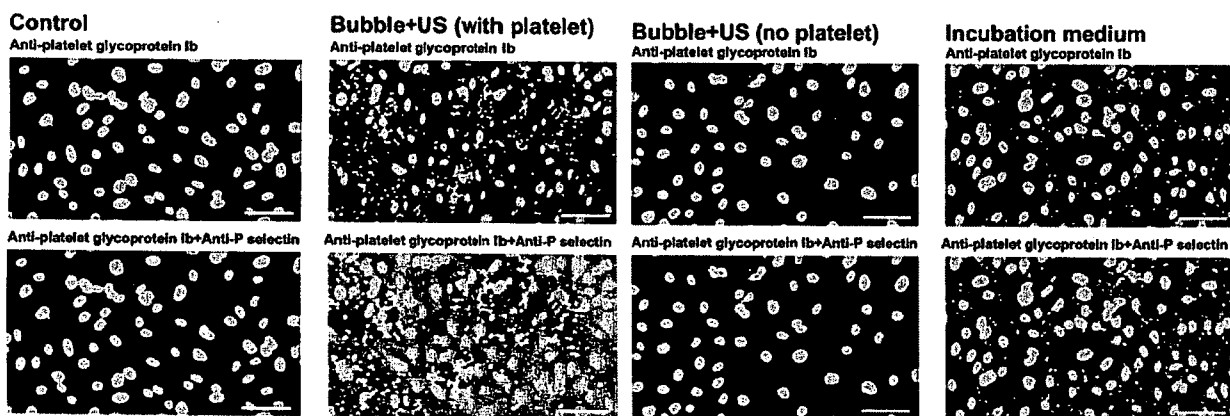
incubation medium, in which platelet-including medium was stimulated by Bubble+US, on the induction of adhesion molecules. The addition of incubation medium caused the apparent increase in P-selectin expression on HUVECs (red fluorescence, right panel in Figure 4), suggesting that the factors released from the platelets stimulated by Bubble+US are closely involved in the induction of adhesion molecules on HUVECs. Treatment of HUVECs by US alone without microbubbles in the medium including platelets did not induce any expression of adhesive molecules (data not shown). These findings suggest that activation of platelets by Bubble+US and release of platelet-derived proinflammatory factors play a key role in the induction of adhesion molecules in the endothelial cells.

### Laminar Flow Assay

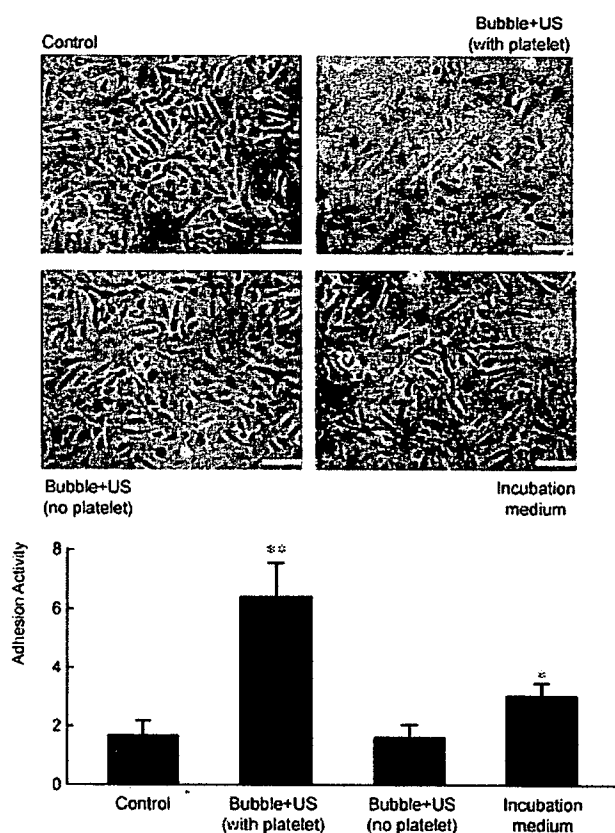
We next studied whether the adhesive activity of BM-MNCs on the endothelium was actually modulated by Bubble+US-activated platelets under laminar flow condition. HUVECs were stimulated by Bubble+US in the medium containing platelets and then the adhesion ratio of BM-MNCs on HUVECs was evaluated under laminar flow as previously reported.<sup>15</sup> The presence of platelets in the medium markedly increased the adhesion ratio of BM-MNCs (3.5-fold,  $P<0.01$ ) compared with Bubble+US without platelets (Figure 5). Moderate increase (1.9-fold,  $P<0.01$ ) was observed in the incubation medium group (in which medium including platelets was stimulated by Bubble+US and then added to HUVECs) compared with the ratio in the untreated HUVECs (control). Treatment of HUVECs by US stimulation without microbubbles in the medium including platelets or addition of control medium including platelet alone (without US stimulation) did not cause a significant increase in the adhesion ratio of BM-MNCs compared with the ratio in the untreated HUVEC (data not shown).

### Discussion

Angiogenic cell therapy by intramuscular implantation of autologous BM-MNCs was shown to be feasible in patients with ischemic limbs.<sup>2</sup> Because intramuscular implantation is



**Figure 4.** Adhesion of platelets and induction of adhesion molecules on Bubble+US-stimulated HUVECs. The presence of platelets and expression of P-selectin are shown by immunostaining with anti-GP-Ib (green fluorescence) and anti-P-selectin (red fluorescence) antibodies and DAPI staining (blue fluorescence). The expression of P-selectin and adhesion of platelets were observed on HUVECs, when HUVECs were stimulated by Bubble+US in the medium containing 10% platelets or exposed to the incubation medium (in which 10% platelet-including medium was stimulated by Bubble+US). The expression of P-selectin (red fluorescence) on HUVECs and attaching platelets was markedly induced. Bar=50  $\mu$ m.



**Figure 5.** Lamellar flow assay of BM-MNCs adhesion. Adhesive activities of BM-MNCs were quantified on HUVEC monolayer under laminar flow. Adhesive cells are indicated by yellow arrows. Cells were considered to be adherent after 10 seconds of stable contact with the monolayer. HUVECs were stimulated by Bubble+US (1MHz, 1.5W, 30 seconds) in the medium with 10% platelets (n=6), without platelets (n=6), or in the incubation medium (in which 10% platelet-including medium was stimulated by Bubble+US, n=6). \* $P < 0.05$ , \*\* $P < 0.01$  vs the untreated control. Bar=100  $\mu\text{m}$ .

invasive at the injected sites, the development of a noninvasive cell delivery system that can target vascular endothelium would be a great advantage for the manipulation of angiogenic cell therapy. A new delivery system of drugs or genes has been developed using US-targeted microbubble destruction; the drugs or genes that attach onto gas-filled microbubbles circulate through the intravascular space and are mechanically destroyed within the target organ by ultrasound,<sup>5-11</sup> whereas no studies were reported to determine whether this method is feasible for delivering the "cells" to specific vascular sites.

US-targeted microbubble destruction was reported to cause an inflammatory action on the cell surface by making small holes that revert to a normal appearance within 24 hours.<sup>10,11</sup> Song et al have reported that US-targeted microbubble destruction causes capillary rupturing that stimulates arteriogenesis and an increase in blood flow in both normal<sup>12</sup> and ischemic<sup>13</sup> skeletal muscles, in which angiogenesis response is transient and unlikely contributes to chronic restoration of blood flow. They concluded that arteriogenesis response rather than angiogenesis plays a major role in US+microbubble-stimulated blood flow recovery. We previously demonstrated that the recruitment of BM-MNCs and platelets stimulates angiogenesis response in the

ischemic muscles by releasing potent angiogenic factors, such as VEGF or bFGF, and supply of endothelial progenitors.<sup>3,14</sup> Furthermore, we have recently reported that systemically transplanted BM-MNCs can be firmly attached onto the injured vascular endothelium in an adhesive molecule-dependent manner.<sup>15</sup> We therefore expanded the previous studies by Song et al<sup>12,13</sup> and examined whether US-targeted microbubble destruction combined with intravenous transplantation of BM-MNCs causes both angiogenesis and arteriogenesis response in an ischemic hindlimb model, leading to a greater enhancement of blood flow restoration.

We found that (1) intravenous infusion of BM-MNCs combined with US-mediated destruction of microbubbles markedly enhances the restoration of regional blood perfusion in ischemic hindlimbs by stimulating both chronic angiogenesis and arteriogenesis response, and (2) release of platelet-derived proinflammatory factors activated by US-mediated destruction of microbubbles causes the adhesion of transfused BM-MNCs on endothelium by inducing the expression of adhesion molecules (P-selectin and ICAM-1). We found that BM-MNCs transfused intravenously were trapped by the spleen and a few BM-MNCs were present in the renal tubules. Considering that BM-derived hematopoietic stem cells were reported to transdifferentiate to renal tubular cells and improve renal function in the ischemia-reperfusion injury model,<sup>19</sup> the present study establishes for the first time that targeted delivery of BM-MNCs by US destruction of microbubbles is an efficient cell delivery system for therapeutic angiogenesis and arteriogenesis, and that the presence of platelets and/or platelet-derived proinflammatory factors activated by US+microbubbles play an important role in the targeted adhesion of BM-MNCs on vascular endothelium.

Previous studies have indicated that platelet attachment to an inter- or sub-endothelial matrix of endothelial cells promotes selectin-mediated leukocyte adhesion to the damaged endothelium under the flow assay condition.<sup>20,21</sup> Inhibition of P-selectin caused a marked inhibition of leukocyte adhesion at a high shear stress.<sup>20</sup> P-selectin is a receptor for leukocytes and monocytes when its expression is induced on activated platelets and endothelium. This property facilitates rapid adhesion of leukocytes to endothelium in injury tissue regions and enhances platelet-leukocyte interactions at sites of inflammation. Endothelial P-selectin is located on membranes of Weibel-Plade bodies, the secretory granules of endothelium in which large multimers of von Willebrand factor (vWF) are stored.<sup>22</sup> After cellular stimulation with agonists such as thrombin or histamine, P-selectin is rapidly expressed on the endothelial cell surface, making it an excellent candidate for directing adherence of unstimulated leukocytes toward endothelium within minutes of tissue injury.<sup>23</sup> Furthermore, we have reported that BM-MNCs have a higher rolling and adhesive activities because of the greater expressions of adhesive molecules such as P-selectin compared with peripheral blood-derived leukocytes.<sup>15</sup> In this study, we found that factors released from platelets stimulated by microbubble destruction are responsible for the attachment of platelets and BM-MNCs onto the endothelium and the induction of endothelial P-selectin and ICAM-1. The present data from the flow assay also confirm that platelet-derived factors play a key role for adhesion of BM-MNCs onto the endothelial cells under

laminar flow. Although we previously showed that platelet-derived VEGF is mainly associated with the angiogenesis response by platelet implantation,<sup>3</sup> preincubation with antibodies for VEGF, bFGF, or PDGF-BB showed no influence on the induction of endothelial P-selectin and ICAM-1 (unpublished observation). Further studies will be required to identify the platelet-derived proinflammatory cytokines responsible for induction of endothelial adhesion molecules. Taken together, these findings suggest that release of platelet-derived proinflammatory factors and direct interaction of platelet onto the endothelial matrix, initiated by US-microbubble destruction, is an underlying mechanism for adhesion of the transfused BM-MNCs on the endothelium under shear stress.

The previous studies showed that US-mediated destruction of microbubbles induces arteriogenesis response in the skeletal muscle, whereas angiogenesis response is transient and unlikely contributes to the increase in the regional blood flow.<sup>12</sup> The arteriogenesis response consists of the formation of new arterioles, which presumably occurs when preexisting capillaries acquire SM coating, and an increase in the diameter of these newly formed and/or preexisting arterioles into channels with larger diameters.<sup>13</sup> Compared with angiogenesis formed by capillary sprouting, arteriogenesis is often studied with the use of conventional angiography. Our angiography finding is consistent with the arteriogenesis response, and the immunohistological data suggest the angiogenesis and arteriogenesis response as evaluated by the increases in capillary numbers and SM coated arterioles, respectively. The neocapillary formation was observed in the day 28 samples, suggesting that the angiogenesis is a chronic response in our study. The controversy with the studies by Song et al<sup>12,13</sup> may be attributable to the difference in the used microbubbles (albumin-coated Optison versus phospholipids-coated BR14). Recruitment of monocytes triggered by monocyte chemoattractant protein-1 was shown to induce arteriogenesis in inflammatory ischemic sites.<sup>24</sup> Because BM-MNCs contain monocyte-lineage progenitor cells,<sup>15</sup> it is plausible that recruitment of BM-MNCs contributes to arteriogenesis together with inflammation response by US+microbubble-mediated capillary rupturing.

In conclusion, the present study demonstrates that intravenous transfusion of BM-MNCs combined with US-destruction of microbubbles is an efficient targeted cell delivery system for therapeutic angiogenesis as well as arteriogenesis, in which the release of platelet-derived proinflammatory factors activated by Bubble+US plays a key role in the attachment of transplanted BM-MNCs onto the endothelial layer.

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## Carbon Dioxide–Rich Water Bathing Enhances Collateral Blood Flow in Ischemic Hindlimb via Mobilization of Endothelial Progenitor Cells and Activation of NO-cGMP System

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**Background**—Carbon dioxide–rich water bathing has the effect of vasodilatation, whereas it remains undetermined whether this therapy exerts an angiogenic action associated with new vessel formation.

**Methods and Results**—Unilateral hindlimb ischemia was induced by resecting the femoral arteries of C57BL/6J mice. Lower limbs were immersed in CO<sub>2</sub>-enriched water (CO<sub>2</sub> concentration, 1000 to 1200 mg/L) or freshwater (control) at 37°C for 10 minutes once a day. Laser Doppler imaging revealed increased blood perfusion in ischemic limbs of CO<sub>2</sub> bathing (38% increase at day 28,  $P < 0.001$ ), whereas N<sup>G</sup>-nitro-L-arginine methyl ester treatment abolished this effect. Angiography or immunohistochemistry revealed that collateral vessel formation and capillary densities were increased (4.1-fold and 3.7-fold,  $P < 0.001$ , respectively). Plasma vascular endothelial growth factor (VEGF) levels were elevated at day 14 (18%,  $P < 0.05$ ). VEGF mRNA levels, phosphorylation of NO synthase, and cGMP accumulation in the CO<sub>2</sub>-bathed hindlimb muscles were increased (2.7-fold, 2.4-fold, and 3.4-fold, respectively) but not in forelimb muscles. The number of circulating Lin<sup>−</sup>/Flk-1<sup>+</sup>/CD34<sup>−</sup> endothelial-lineage progenitor cells was markedly increased by CO<sub>2</sub> bathing (24-fold at day 14,  $P < 0.001$ ). The Lin<sup>−</sup>/Flk-1<sup>+</sup>/CD34<sup>−</sup> cells express other endothelial antigens (endoglin and VE-cadherin) and incorporated acetylated LDL.

**Conclusions**—Our present study demonstrates that CO<sub>2</sub> bathing of ischemic hindlimb causes the induction of local VEGF synthesis, resulting in an NO-dependent neocapillary formation associated with mobilization of endothelial progenitor cells. (*Circulation*. 2005;111:1523-1529.)

**Key Words:** carbon dioxide ■ hypercapnia ■ angiogenesis ■ stem cells ■ endothelium ■ vasculogenesis

Carbon dioxide–rich (CO<sub>2</sub>) water bathing has a long history and is thought to be effective in the treatment of peripheral vascular disorder<sup>1</sup>; however, the mechanism(s) underlying this traditional therapy remains poorly defined. The effect of CO<sub>2</sub>-enriched water on cutaneous circulation depends primarily on the vasodilatation elicited by the CO<sub>2</sub> that diffuses into the subcutaneous tissue through the skin layers.<sup>2,3</sup> Findings in the intact coronary circulation<sup>4</sup> and in isolated aortic strips<sup>5</sup> have suggested that vasodilatation in response to CO<sub>2</sub> may be mediated in part by nitric oxide (NO).

Previous investigations have provided inferential evidence that biological processes modulated by NO might extend to include angiogenesis. Direct in vitro evidence that NO may induce angiogenesis was demonstrated recently by Papapetropoulos et al.<sup>6,7</sup> Ziche et al<sup>8,9</sup> established the first line of evidence that NO can induce angiogenesis in vitro. Murohara et al<sup>10</sup> clearly showed NO-mediated angiogenesis in response to tissue ischemia in NO-deficient mice. We have also reported that overexpression of endothelial NO synthase (eNOS) causes a marked increase in

neocapillary formation in response to tissue ischemia.<sup>11</sup> Furthermore, hypercapnia-associated acidosis was reported to induce the expression of angiogenic factors, vascular endothelial growth factor (VEGF), or basic fibroblast growth factor and inhibit endothelial cell apoptosis.<sup>12</sup> Taken together, this accumulated evidence may raise the possibility that the CO<sub>2</sub>-enriched water bathing therapy enhances regional blood perfusion by increasing new vessel formation. In the present study, we report that CO<sub>2</sub>-enriched water bathing stimulates blood flow restoration in the ischemic hindlimbs of mice by increasing NO-dependent collateral vessel formation and the mobilization of endothelial-lineage progenitor cells into the circulation.

### Methods

#### Principle of the Device

This device uses a CO<sub>2</sub> gas–permeable membrane similar to the principle of an artificial lung on the extracorporeal circulatory system. The unit consists of 15 000 multilayered composite-membrane hollow fibers with porous membrane sandwiching on

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both sides of gas-permeable membrane (Mitsubishi-Leiyon) and is capable of instantly converting 20 L/min of water (pH 7.0) into CO<sub>2</sub>-enriched water (free CO<sub>2</sub> concentration, 1000 to 1200 mg/L, pH 5.0).

### Mouse Model of Unilateral Hindlimb Ischemia and CO<sub>2</sub> Bathing

Unilateral hindlimb ischemia was induced by resecting the right femoral arteries (including muscle branches) and veins of 8-week-old male C57BL/J mice under anesthesia with sodium pentobarbital (50 mg/kg IP).<sup>11,13</sup> To inhibit NOS chronically, the mice were provided water containing 1 mg/mL N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) for 4 weeks.<sup>11</sup> Because CO<sub>2</sub> bathing immediately after operation delayed the closure of this skin wound, we started the CO<sub>2</sub> bathing of the lower limb from 4 days after surgery. Lower limbs of mice were immersed into CO<sub>2</sub>-enriched water for 10 minutes or freshwater (control) at 37°C once a day under anesthesia (n=10 in each group). The Institutional Animal Care and Use Committee of our university approved all animal protocols.

### Immunohistochemistry

Four pieces of ischemic tissues from the adductor and semimembranosus muscles were obtained 28 days after the surgery of hindlimb ischemia. Frozen sections were stained with anti-factor VIII, followed by incubation with TRIC-conjugated secondary antibody. Five fields from 2 muscle samples of each animal were randomly selected for capillary counts. To ensure that capillary densities were not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was determined.<sup>11,13</sup> To examine whether cells survived in the tissues, adjacent sections were subjected to alkaline phosphatase staining by the indoxyl-tetrazolium method. Alkaline phosphatase staining turns capillary endothelial cells a dark blue color only when they are viable and when the intracellular enzyme activity remains intact.<sup>11,13</sup>

### Laser Doppler Analysis and Angiography

We measured the ratio of the ischemic (right)/normal (left) limb blood flow by use of a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments). After blood flow had been scanned twice, stored images were subjected to computer-assisted quantification of blood flow, and average flows of the ischemic and nonischemic limbs were calculated. To minimize data variables caused by ambient light and temperature, the LDPI index was expressed as the ratio of ischemic (left) to nonischemic (right) limb blood flow.<sup>11,13</sup>

Vessel density was evaluated with a microfocus x-ray television device (Hitex Co Ltd) 28 days after ischemia (n=5). Longitudinal laparotomy was performed to introduce a catheter into the abdominal aorta, followed by injection of contrast medium (lipiodol). Angiography was performed for 2 seconds after the injection. We quantitatively analyzed collateral vessel numbers as previously reported.<sup>11,13</sup> Briefly, numbers of vessels in the thigh area were counted by use of 5-mm<sup>2</sup> grids by 2 radiologists who were unaware of the group identity of the angiographic film. Interobserver variation was <5%.

### cGMP Assay and Measurement of Blood pH Level

The assay for tissue cGMP was performed by use of the cGMP enzyme immunoassay system (Biotrak; Amersham) as previously described.<sup>11</sup> The tissues remaining after cGMP measurement were digested by use of a bicinchoninic acid protein assay kit (Pierce). Blood pH levels were measured by automated blood gas analyzer (ABL505, Radiometer A/S).

### Northern and Western Blotting and Plasma VEGF Measurement

Frozen skeletal samples from hindlimbs or forelimbs were homogenized in Trizol reagent (Gibco BRL). Blots were hybridized with a random-primed <sup>32</sup>P-labeled cDNA probe for VEGF<sup>11</sup> and normalized

by densities for GAPDH as an internal control. Hybridized signals were measured by scanning densitometry, and VEGF mRNA levels were arbitrarily normalized relative to the GAPDH mRNA levels.

Phosphorylation of eNOS (serine 1177) was analyzed by Western blotting using phospho-specific antibodies (New England Biolabs). The muscles were homogenized in lysis buffer. Lysates were immunoblotted with anti-phospho antibodies and detected with an enhanced chemiluminescence kit (Amersham).<sup>11</sup> Plasma VEGF concentration was measured by use of the ELISA kit (R&D Systems).

### FACS Staining

Total nuclear cells in the peripheral blood were isolated by erythrocyte lysis with ammonium chloride solution (PharM Lyse, Becton Dickinson). Lin<sup>-</sup>/Flk<sup>+</sup> cells were isolated by PE-labeled lineage antigens (CD11b, CD3, B220, Ter-199, Gr-1, CD4, CD8e, CD16/32), FITC-CD34, and biotin-Flk-1 and then analyzed by use of a FACScan flow cytometer.<sup>14,15</sup> Lin<sup>-</sup>/Flk<sup>+</sup>/endoglin<sup>+</sup> cells were isolated by FITC-labeled lineage antigens, PE-Flk-1 and biotin-endoglin. To prove the specificity of anti-CD34 antibody, the biotin-labeled anti-mouse CD34 antibody used in this study was reacted with mouse bone marrow cells and purified with streptavidin-magnet beads, followed by fluorescence-activated cell sorter (FACS) analysis using streptavidin-PE. All anti-mouse antibodies were purchased from BD Biosciences.

### Differentiation of Lin<sup>-</sup>/Flk-1+ Cells Into Endothelial Cells In Vitro

The population of Lin<sup>-</sup>/Flk-1+ cells was isolated with FACS from the peripheral blood of the mice that had undergone the limb ischemic operation and then treated with CO<sub>2</sub> bathing for 14 days. These cells were cultured on fibronectin-coated plastic dishes in DMEM supplemented with 100 ng/mL VEGF and 10% FBS. After 4 days, DiI-labeled acetylated LDL (Biomedical Technologies Inc) was added into medium at 2 μg/mL for 6 hours, fixed with 4% paraformaldehyde, and stained with anti-VE-cadherin antibody and FITC-labeled anti-IgG antibody.

### Statistics

Statistical analyses were performed by 1-way ANOVA followed by pairwise contrasts using Dunnett's test. Data (mean±SEM) were considered significant at a value of *P*<0.05.

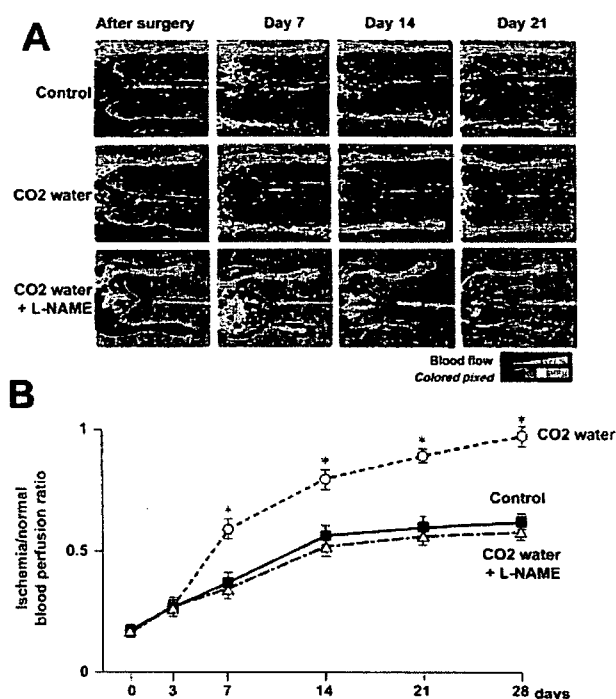
## Results

### Laser Doppler Blood Perfusion

Progressive recovery of limb perfusion was disclosed in CO<sub>2</sub>-bathed and control freshwater-bathed mice after induction of limb ischemia. A greater degree of blood perfusion recovery was observed in the ischemic limbs of CO<sub>2</sub>-bathed mice compared with controls (38% increase at day 28, *P*<0.001) (Figure 1, A and B). Inhibition of NOS activity by L-NAME administration abolished an enhancement of blood flow recovery by CO<sub>2</sub> bathing and reversed the recovery ratio toward the control level. Blood flow in L-NAME-treated mice tended to be lower than that in wild-type mice, but this difference was not significant (Figure 1B).

### Angiography

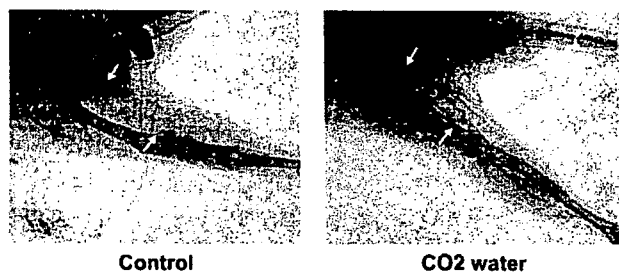
All animals were subjected to iliac angiography using contrast medium (lipiodol) on postoperative day 28. Collateral vessel numbers were markedly increased in ischemic limbs of CO<sub>2</sub>-bathed mice (4.1±0.4-fold at day 28, *P*<0.001, n=5) compared with those in water-bathed mice (Figure 2).



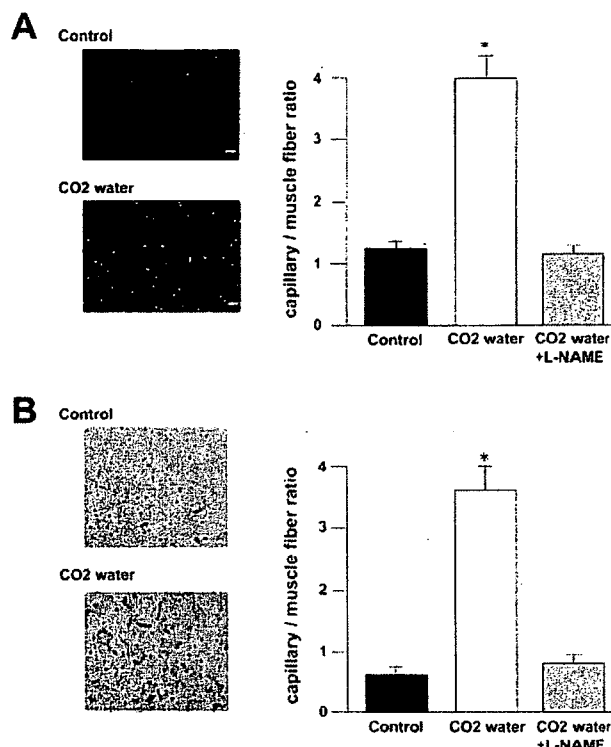
**Figure 1.** LDPI. A, Greater blood perfusion (red to yellow) was observed in CO<sub>2</sub>-enriched water-bathed limbs, in contrast to reduced perfusion (green to blue) in freshwater-bathed ischemic limbs (control). B, Computer-assisted analyses of LDPI revealed significantly greater blood perfusion values in CO<sub>2</sub>-enriched water-bathed group than in control group. Administration of L-NAME (1 mg/mL) in drinking water reduced increased perfusion by CO<sub>2</sub>-enriched water bathing toward normal level. Values shown are mean ± SEM (n=10) at each time point. \*P<0.001 vs control mice.

**Analysis of Capillary Density**

Immunohistochemical staining for anti-factor VIII revealed the presence of capillary endothelial cells (Figure 3A). The capillary/muscle fiber ratio in the skeletal muscle obtained 28 days after hindlimb ischemia was significantly increased in the CO<sub>2</sub>-bathed mice (3.7-fold, P<0.001) compared with that in water-bathed mice. A similar increase (4.2-fold increase, P<0.001) was also observed in ALP staining for detection of viable endothelial cells (Figure 3B). Administration of L-NAME (1 mg/mL) in drinking water reduced the increased



**Figure 2.** Angiographic analysis. Representative angiograms were obtained on postoperative day 28. Arrows indicate ligated ends of femoral arteries. Collateral vessel numbers counted by use of 5-mm<sup>2</sup> grids were markedly increased in ischemic limbs of CO<sub>2</sub>-bathed mice (4.1 ± 0.4-fold at day 28, P<0.001, n=5) compared with those in water-bathed mice.



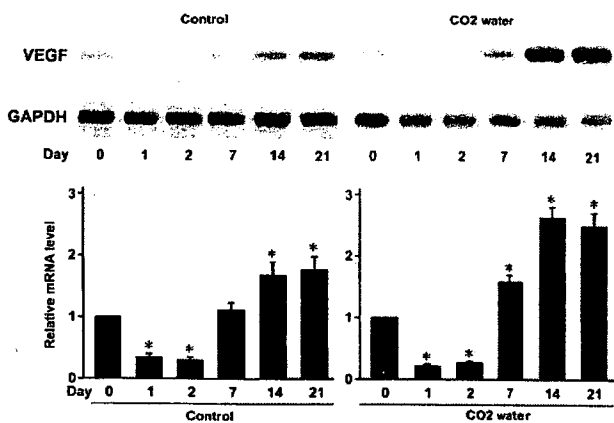
**Figure 3.** Immunohistochemical analysis. A, Ischemic tissues from adductor and semimembranosus muscles were obtained 28 days after surgery of hindlimb ischemia. Endothelial cells were stained with anti-factor VIII antibody, followed by incubation with TRIC-conjugated secondary antisera. B, Alkaline phosphatase staining turns viable endothelial cells blue. Five fields from 2 muscle samples of each animal (n=10) were randomly selected, and capillary density was shown as capillary/muscle fiber ratio. Administration of L-NAME (1 mg/mL) in drinking water reduced increased vessel numbers by CO<sub>2</sub> bathing toward control levels of freshwater-bathed ischemic limbs (n=10). \*P<0.001 vs control mice. Bars=50 μm.

vessel numbers by CO<sub>2</sub> bathing toward the normal level (Figure 3).

**Induction of VEGF Expression, eNOS Phosphorylation, and cGMP Levels**

VEGF mRNA levels were examined in hindlimb muscles dissected at days 0 (before), 1, 2, 7, 14, and 21. VEGF mRNA levels were decreased immediately after hindlimb ischemia (day 1, day 2), and then gradually reverted to the basal levels at day 7 in the control group. In the CO<sub>2</sub>-enriched water group, a marked increase in VEGF mRNA levels was observed at day 7 (1.6-fold versus day 0 preischemic levels, P<0.01) and showed a peak level at ≈day 14 (2.7-fold versus day 0, P<0.001). Induction of the VEGF mRNA from the preischemic level was significantly higher in the CO<sub>2</sub> bathing group than the increase in the control group (Figure 4), whereas the increase in VEGF mRNA synthesis by CO<sub>2</sub> bathing was not affected by L-NAME treatment (Figure 5A).

To define whether the effect of CO<sub>2</sub> bathing results from systemic or local VEGF synthesis, we examined the time-dependent VEGF mRNA induction in forelimb skeletal muscles after CO<sub>2</sub> bathing and changes in plasma VEGF levels. The mRNA levels in forelimb skeletal muscles of CO<sub>2</sub>-bathed



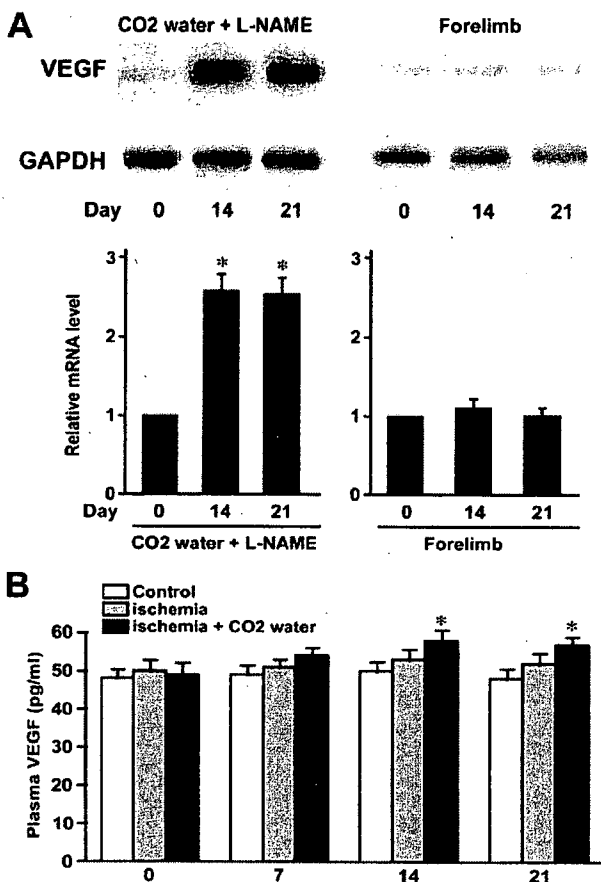
**Figure 4.** VEGF mRNA expression in ischemic hindlimb. Hindlimb skeletal muscles were dissected after ischemia, and RNA was extracted. Densities of VEGF mRNA signals were measured by densitometry and normalized relative to those of GAPDH mRNA signals. Results (mean $\pm$ SEM, n=6) were arbitrarily indicated as values relative to VEGF mRNA levels at day 0. \* $P$ <0.001 vs day 0 preischemic levels.

ischemic mice did not change significantly after CO<sub>2</sub> bathing compared with the preischemic levels (Figure 5A). Although only induction of hindlimb ischemia did not affect plasma VEGF levels, VEGF levels in the CO<sub>2</sub>-bathed ischemic mice were slightly but significantly elevated at day 14 (18%,  $P$ <0.05, n=6) compared with the water-immersed ischemic mice (n=6) (Figure 5B). Furthermore, we determined plasma pH levels to study whether CO<sub>2</sub> bathing-mediated effects are systemic. We found that CO<sub>2</sub> bathing of ischemic lower limbs did not significantly affect the pH levels in the peripheral blood (control, 7.2 $\pm$ 0.04; CO<sub>2</sub> bathing, 7.2 $\pm$ 0.03 at day 14; n=6 each). These findings demonstrate that VEGF synthesis by CO<sub>2</sub> bathing is induced only locally and that this increase in local VEGF synthesis leads to the elevation in plasma VEGF levels.

Skeletal muscles at day 14 (in which VEGF expression is maximally increased) were dissected, and eNOS phosphorylation and cGMP levels were examined. The eNOS phosphorylation levels at day 14 (normalized with expression levels of eNOS protein) were increased significantly in both control and CO<sub>2</sub>-enriched water groups relative to the day 0 preischemic levels (1.6- and 2.4-fold, respectively) (Figure 6A). eNOS phosphorylation levels in the CO<sub>2</sub>-enriched water group were significantly higher than those in the control group ( $P$ <0.001 versus the control group). Consistent with eNOS phosphorylation, cGMP levels in skeletal muscles at day 14 were also significantly higher (3.4-fold,  $P$ <0.001) in the CO<sub>2</sub>-enriched water group compared with those in the control group (Figure 6B).

#### Effect of CO<sub>2</sub> Bathing on Circulating Endothelial-Lineage Progenitor Cells

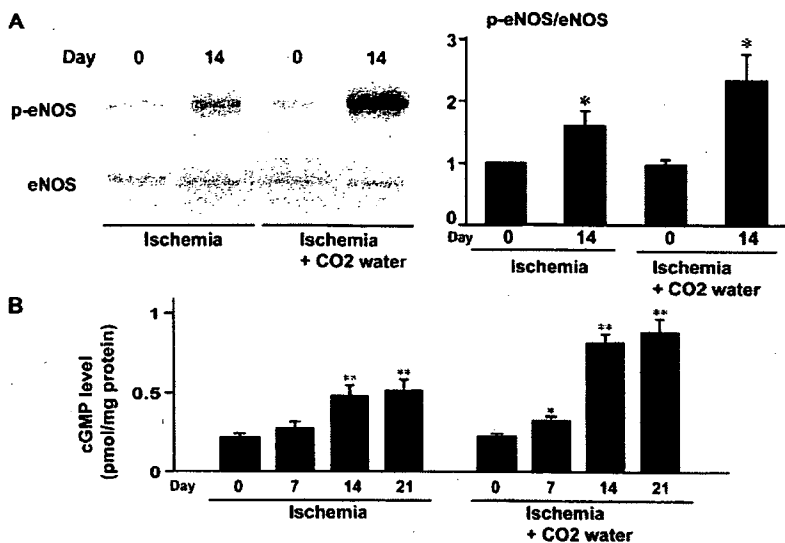
CD34<sup>+</sup>/AC133<sup>+</sup>/Flk-1<sup>+</sup> hematopoietic stem cells circulate in the peripheral blood of humans as an endothelial precursor cell and play a critical role in neovascularization in ischemic tissue.<sup>16</sup> Because AC133 marker is not available for mice, we isolated hematopoietic lineage-negative (Lin<sup>-</sup>) cells from the peripheral blood and then analyzed the CD34<sup>-</sup> and endothe-



**Figure 5.** Effect of L-NAME on VEGF mRNA expression in hindlimb and forelimb skeletal muscles after hindlimb ischemia and plasma VEGF concentrations. A, Mice with hindlimb ischemia were provided water containing 1 mg/mL L-NAME, and time-dependent VEGF mRNA expression was measured as described in Figure 4. Forelimb skeletal muscles were dissected from hindlimb ischemia mice, and VEGF mRNA was analyzed. Results (mean $\pm$ SEM, n=5) were arbitrarily indicated as values relative to VEGF mRNA levels at day 0. \* $P$ <0.001 vs day 0 preischemic levels. B, Plasma VEGF concentrations were measured by ELISA (control, CO<sub>2</sub> bathing of normal mice; ischemia, freshwater-bathed ischemic mice; ischemia+CO<sub>2</sub> water, CO<sub>2</sub>-bathed ischemic mice, n=5 each). \* $P$ <0.05 vs day 0.

lial markers Flk-1- and endoglin-positive population to study whether endothelial-lineage precursor cells are mobilized by CO<sub>2</sub>-enriched water bathing. FACS analysis indicated that Lin<sup>-</sup>/Flk-1<sup>+</sup> cells are barely detected in the peripheral blood of normal mice (0.01 $\pm$ 0.002% of total nuclear cells, n=12). Lin<sup>-</sup>/Flk-1<sup>+</sup> cells were significantly increased after limb ischemia and showed a peak value at day 14 ( $\approx$ 7-fold versus the preischemic value) (from 0.01 $\pm$ 0.002% to 0.073 $\pm$ 0.002%,  $P$ <0.001, n=7). Interestingly, such a Lin<sup>-</sup>/Flk-1<sup>+</sup> population was further increased by CO<sub>2</sub> bathing and showed a peak value at day 14 ( $\approx$ 24-fold increase, from 0.01 $\pm$ 0.002% to 0.24 $\pm$ 0.03%,  $P$ <0.001, n=7) (Figure 7A). The Lin<sup>-</sup>/Flk-1<sup>+</sup> cells mobilized by CO<sub>2</sub> bathing were mostly positive for anti-endoglin antibody and in the CD34<sup>-</sup> fraction (Figure 7B). Considering that CD34<sup>-</sup>/Flk-1<sup>+</sup> cells rather than CD34<sup>+</sup>/Flk-1<sup>+</sup> are reported to be a real population of hematopoietic stem cells,<sup>17</sup> our present data





**Figure 6.** cGMP accumulation in ischemic limbs. A, Skeletal muscles were homogenized and immunoblotted with anti-phospho antibodies for eNOS. Phospho signals in filters were stripped and reprobed with anti-eNOS antibody. Phospho-eNOS densities were measured by densitometry and normalized relative to those of eNOS signals. Results are arbitrarily indicated as values relative to signal densities in day 0 preischemic control. Results shown are mean  $\pm$  SEM (n=6), and representative data are shown. \* $P < 0.001$  vs values in day 0 preischemic control. B, Skeletal muscles were dissected at days 7, 14, and 21 after hindlimb ischemia, and tissue cGMP levels were measured. Results shown are mean  $\pm$  SEM (n=6 each). \* $P < 0.05$ , \*\* $P < 0.001$  vs day 0 preischemic control.

indicate that CO<sub>2</sub>-enriched water bathing mobilizes very immature hematopoietic stem cells, including endothelial progenitor cells. To prove the specificity of anti-CD34 antibody, CD34<sup>+</sup> cells were enriched by anti-mouse CD34 antibody from mouse bone marrow cells. As shown in Figure 7C, 83% purity of CD34-positive cells was detected by FACS, indicating that the staining for the CD34 antigen was properly performed.

We further examined whether Lin<sup>-</sup>/Flk-1<sup>+</sup> cells express another endothelial marker, VE-cadherin, and also possess the characteristics of endothelial cells, such as acetylated LDL uptake. The Lin<sup>-</sup>/Flk-1<sup>+</sup> cell population, mobilized by CO<sub>2</sub> bathing, was isolated and cultured with 100 nmol/L VEGF-supplemented medium for 4 days. Approximately 26  $\pm$  1.2% (n=12) of the Lin<sup>-</sup>/Flk-1<sup>+</sup> cells adhered onto the fibronectin-coated plastic dishes. Approximately 74  $\pm$  2.3% (n=12) of the attaching cells showed the ability to incorporate the DiI-labeled acetylated LDL, and these cells expressed the VE-cadherin (Figure 7D).

### Discussion

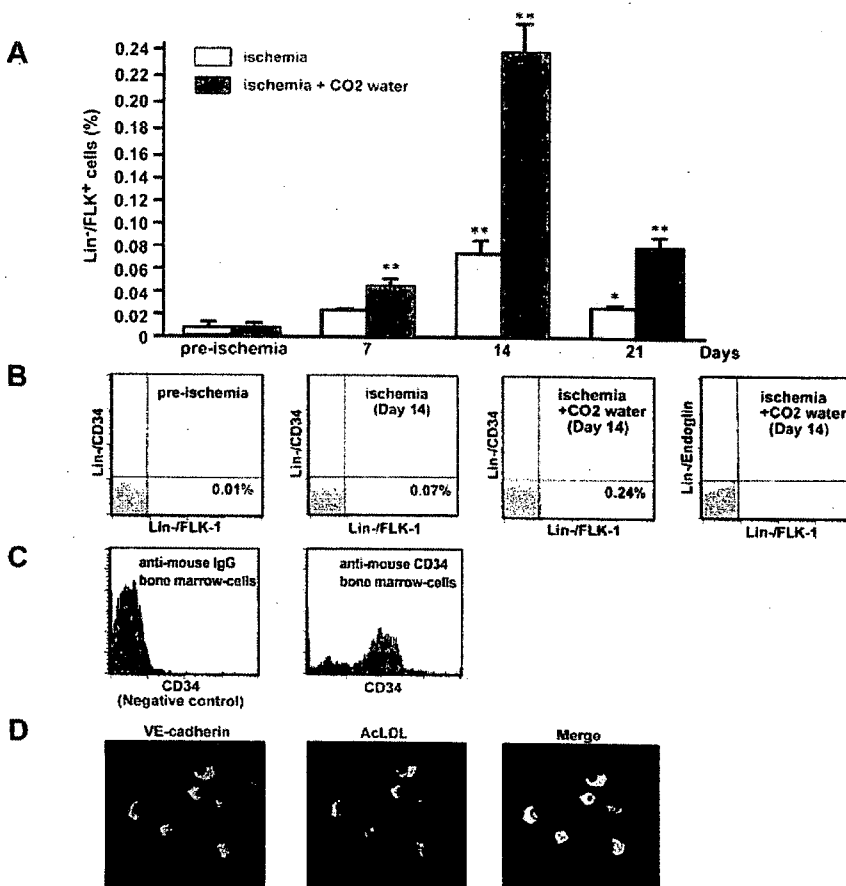
A number of reports about the physiological effects of CO<sub>2</sub>-enriched water on subcutaneous microcirculation have been published. Savin et al<sup>18</sup> reported that transfer of CO<sub>2</sub> across the skin can have beneficial local vasomotor effects. Hartmann et al<sup>19</sup> demonstrated an increase in tissue oxygen brought about by the Bohr effect in addition to the vasodilation effect by CO<sub>2</sub> or vasodilation by decrease in plasma catecholamine levels. Toriyama et al<sup>20</sup> also reported that the effect of CO<sub>2</sub>-enriched water on the subcutaneous microcirculation results from peripheral vasodilation resulting from increased parasympathetic and decreased sympathetic nerve activity. Findings in the intact coronary circulation<sup>4</sup> and in isolated aortic strips<sup>5</sup> have suggested that vasodilation in response to CO<sub>2</sub> may be mediated in part by NO. Consistent with these previous studies, our present study demonstrates that immersion of ischemic hindlimb into CO<sub>2</sub>-enriched water bathing causes an NO-dependent increase in collateral blood perfusion, induction of regional VEGF synthesis, and mobi-

lization of endothelial-lineage progenitor cells into the circulation.

What is the mechanism responsible for the proangiogenic effect by CO<sub>2</sub>-enriched water bathing? In the endothelial cells cultured in the medium equilibrated with hypercapnia-associated acidosis, the expressions of potent angiogenic factors, such as VEGF or basic fibroblast growth factor, are increased and endothelial cell apoptosis is inhibited.<sup>12</sup> VEGF was well known to mobilize endothelial progenitor cells from bone marrow into the circulation.<sup>21</sup> Our present data clearly indicate that VEGF expression is markedly induced in hindlimb skeletal muscles after CO<sub>2</sub>-enriched water bathing. A high concentration of CO<sub>2</sub> (1000 to 1200 mg/L) liberates free CO<sub>2</sub> in the freshwater (pH 7.0), resulting in a decrease in pH level (pH 5.0). We found that CO<sub>2</sub> bathing of ischemic lower limbs did not affect the pH levels in the peripheral blood and VEGF mRNA synthesis in the forelimb muscle. Considering that acidosis induces VEGF expression in the endothelial cells,<sup>12</sup> the local tissue acidosis by CO<sub>2</sub> bathing, rather than the CO<sub>2</sub> content of the water, may induce VEGF synthesis in the local skeletal muscles. Furthermore, calcium mobilization associated with local tissue pH changes may serve as alternate, or contributory, mechanisms for these observations.

Previous studies reported that VEGF stimulates the release of NO from the arterial wall<sup>22,23</sup> and promotes the recovery of disturbed endothelium-dependent flow in the ischemic hindlimb.<sup>24</sup> Involvement of NO in the angiogenic properties of VEGF has been established in the NO-deficient mice; Murohara et al<sup>10</sup> showed NO-mediated angiogenesis in the hindlimb ischemia model, and Aicher et al<sup>25</sup> reported that VEGF-mediated mobilization of endothelial progenitor cells is reduced in NO-deficient mice. The present study revealed that inhibition of NOS activity by L-NAME inhibited the recovery of collateral blood flow by CO<sub>2</sub> bathing without affecting local VEGF synthesis. Taken together, these findings demonstrate that the proangiogenic effect by CO<sub>2</sub> bathing is a result of activation of NO-mediated signaling and that this activation results from the downstream effects of VEGF. Considering that VEGF-mediated mobilization of endothelial





**Figure 7.** FACS analysis of circulating endothelial-lineage progenitor cells. A and B, Lin<sup>-</sup>/Flk<sup>+</sup> cells in peripheral blood nuclear cells were isolated by PE-labeled lineage antigens, FITC-CD34 and biotin-Flk-1, and then analyzed. Lin<sup>-</sup>/Flk<sup>+</sup>/endoglin<sup>+</sup> cells were isolated by FITC-labeled lineage antigens, PE-Flk-1 and biotin-endoglin. Cell number ratio of Lin<sup>-</sup>/Flk<sup>+</sup> cells to total nuclear cells is shown (n=6 each). \*P<0.05, \*\*P<0.001 vs day 0 preischemic control. C, To prove specificity of anti-CD34 antibody, CD34<sup>+</sup> cells in mouse bone marrow cells were enriched by biotin-labeled anti-mouse CD34 antibody and purified with streptavidin-magnet beads, followed by FACS analysis using streptavidin-PE. Rat anti-mouse IgG was used as a negative control. D, Lin<sup>-</sup>/Flk<sup>+</sup> cells were cultured on fibronectin-coated plastic dishes in DMEM supplemented with 100 ng/mL VEGF and 10% FCS. After 4 days, Dil-labeled acetylated LDL was added into medium at 2  $\mu$ g/mL for 6 hours, fixed with 4% paraformaldehyde, and stained with anti-VE-cadherin antibody and FITC-labeled anti-IgG antibody.

progenitor cells is NO-dependent,<sup>25</sup> our present study suggests that CO<sub>2</sub> bathing causes the induction of local VEGF synthesis, resulting in an NO-dependent neocapillary formation associated with mobilization of endothelial progenitor cells.

Hartman et al<sup>19</sup> reported that repeated CO<sub>2</sub>-enriched water bathing increases arterial flow, transcutaneous oxygen tension, and pain-free walking distance in the clinical trial of peripheral arterial disease. Although they have not performed angiography, the enhancement of neovascularization may cause the increases in walking distance in addition to the improvement of blood vessel function. Toriyama et al<sup>20</sup> also showed that CO<sub>2</sub> foot bathing is clinically effective in the salvage of critical limb ischemia. In conclusion, our present study clearly demonstrates for the first time that CO<sub>2</sub>-enriched water bathing causes the enhanced induction of local VEGF synthesis associated with activation of the NO-cGMP pathway and mobilization of endothelial progenitor cells, resulting in NO-dependent neocapillary formation that leads to an increase in collateral blood flow. Thus, these findings indicate that the CO<sub>2</sub>-enriched water bathing therapy can be included in angiogenic therapies associated with neovascularization, such as the transplantation of bone marrow mononuclear cells<sup>14</sup> or VEGF gene therapy.<sup>26</sup>

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