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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/J mice. Lower limbs were immersed in CO_2-enriched water (CO_2 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_2 bathing (38% increase at day 28, $P<0.001$), whereas N^G-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_2-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−/Flk-1+/CD34− endothelial-lineage progenitor cells was markedly increased by CO_2 bathing (24-fold at day 14, $P<0.001$). The Lin−/Flk-1+/CD34− cells express other endothelial antigens (endoglin and VE-cadherin) and incorporated acetylated LDL.

Conclusions—Our present study demonstrates that CO_2 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_2) water bathing has a long history and is thought to be effective in the treatment of peripheral vascular disorder[1]; however, the mechanism(s) underlying this traditional therapy remains poorly defined. The effect of CO_2-enriched water on cutaneous circulation depends primarily on the vasodilatation elicited by the CO_2 that diffuses into the subcutaneous tissue through the skin layers.[2,3] Findings in the intact coronary circulation[4] and in isolated aortic strips[5] have suggested that vasodilation in response to CO_2 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.[6,7] Ziche et al[8,9] established the first line of evidence that NO can induce angiogenesis in vitro. Murohara et al[10] 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.[11] 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.[12] Taken together, this accumulated evidence may raise the possibility that the CO_2-enriched water bathing therapy enhances regional blood perfusion by increasing new vessel formation. In the present study, we report that CO_2-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_2 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_2-enriched water (free CO_2 concentration, 1000 to 1200 mg/L, pH 5.0).

Mouse Model of Unilateral Hindlimb Ischemia and CO_2 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).[11,13] To inhibit NOS chronically, the mice were provided water containing 1 mg/mL N^G-nitro-L-arginine methyl ester (L-NAME) for 4 weeks.[11] Because CO_2 bathing immediately after operation delayed the closure of this skin wound, we started the CO_2 bathing of the lower limb from 4 days after surgery. Lower limbs of mice were immersed into CO_2-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 semimembranous 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.[11,13] 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.[11,13]

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.[11,13]

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.[11,13] Briefly, numbers of vessels in the thigh area were counted by use of 5-mm^2 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.[11] 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 ^{32}P-labeled cDNA probe for VEGF[11] 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).[11] 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−/Flk+ 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.[14,15] Lin−/Flk+/endoglin+ 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−/Flk-1+ Cells Into Endothelial Cells In Vitro

The population of Lin−/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_2 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_2-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_2-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_2 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_2-bathed mice (4.1±0.4-fold at day 28, $P<0.001$, n=5) compared with those in water-bathed mice (Figure 2).

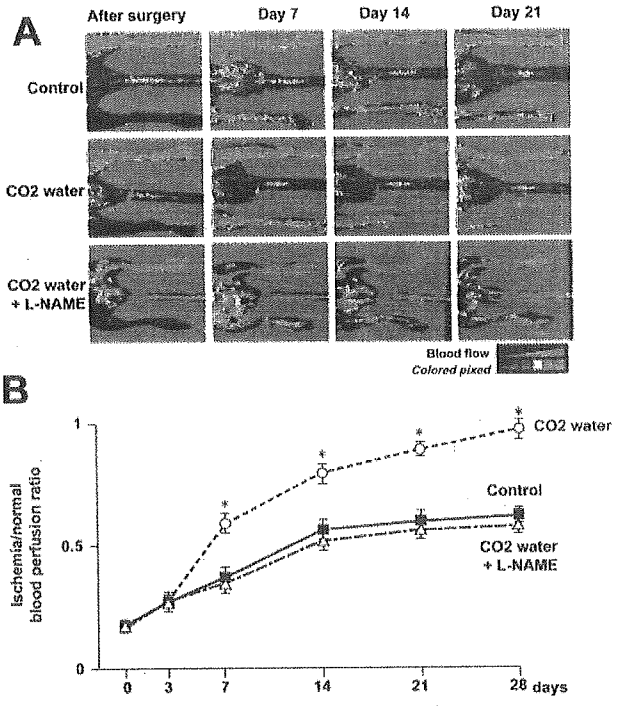

Figure 1. LDPI. A, Greater blood perfusion (red to yellow) was observed in CO_2-enriched water-bathed limbs, in contrast to reduced perfusion (green to blue) in freshwater-bathed ischemic limbs (control). B, Computer-assisted analyses of LDPI revealed significantly greater blood perfusion values in CO_2-enriched water-bathed group than in control group. Administration of L-NAME (1 mg/mL) in drinking water reduced increased perfusion by CO_2-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_2-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

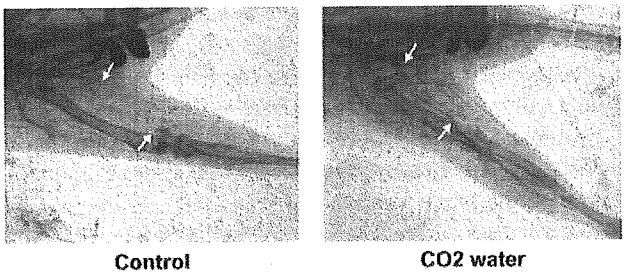

Figure 2. Angiographic analysis. Representative angiograms were obtained on postoperative day 28. Arrows indicate ligated ends of femoral arteries. Collateral vessel numbers counted by use of 5-mm^2 grids were markedly increased in ischemic limbs of CO_2-bathed mice (4.1±0.4-fold at day 28, P<0.001, n=5) compared with those in water-bathed mice.

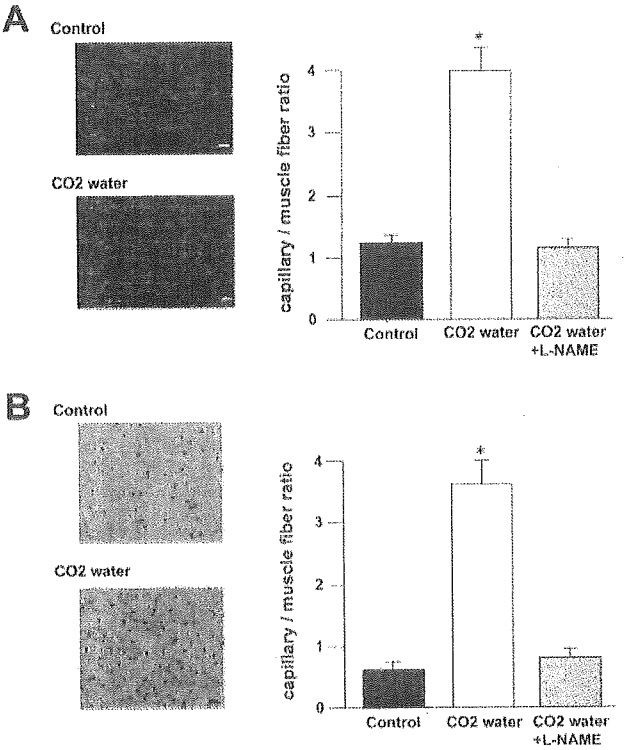

Figure 3. Immunohistochemical analysis. A, Ischemic tissues from adductor and semimembranous muscles were obtained 28 days after surgery of hindlimb ischemia. Endothelial cells were stained with anti-factor VIII antibody, followed by incubation with TRIC-conjugated secondary antisera. B, Alkaline phosphatase 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_2 bathing toward control levels of freshwater-bathed ischemic limbs (n=10). *P<0.001 vs control mice. Bars=50 μm.

vessel numbers by CO_2 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_2-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_2 bathing group than the increase in the control group (Figure 4), whereas the increase in VEGF mRNA synthesis by CO_2 bathing was not affected by L-NAME treatment (Figure 5A).

To define whether the effect of CO_2 bathing results from systemic or local VEGF synthesis, we examined the time-dependent VEGF mRNA induction in forelimb skeletal muscles after CO_2 bathing and changes in plasma VEGF levels. The mRNA levels in forelimb skeletal muscles of CO_2-bathed

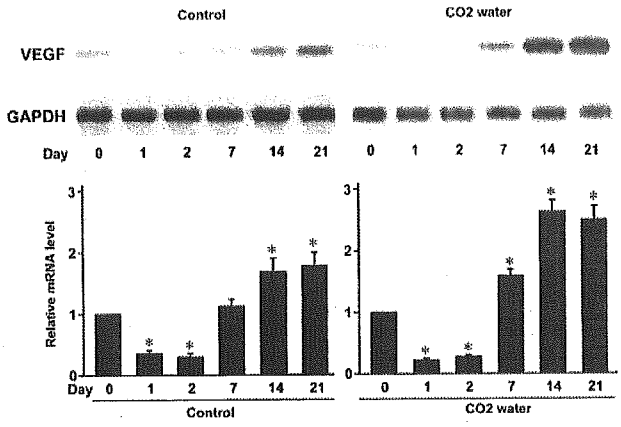

Figure 4. VEGF mRNA expression in ischemic hindlimb. Hindlimb skeletal muscles were dissected after ischemia, and RNA was extracted. Densities of VEGF mRNA signals were measured by densitometry and normalized relative to those of GAPDH mRNA signals. Results (mean±SEM, n=6) were arbitrarily indicated 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_2 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_2-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_2 bathing–mediated effects are systemic. We found that CO_2 bathing of ischemic lower limbs did not significantly affect the pH levels in the peripheral blood (control, 7.2±0.04; CO_2 bathing, 7.2±0.03 at day 14; n=6 each). These findings demonstrate that VEGF synthesis by CO_2 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_2-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_2-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_2-enriched water group compared with those in the control group (Figure 6B).

Effect of CO_2 Bathing on Circulating Endothelial-Lineage Progenitor Cells

CD34+/AC133+/Flk-1+ 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.[16] Because AC133 marker is not available for mice, we isolated hematopoietic lineage-negative (Lin−) cells from the peripheral blood and then analyzed the CD34- and endothelial markers Flk-1– and endoglin-positive population to study whether endothelial-lineage precursor cells are mobilized by CO_2-enriched water bathing. FACS analysis indicated that Lin−/Flk-1+ cells are barely detected in the peripheral blood of normal mice (0.01±0.002% of total nuclear cells, n=12). Lin−/Flk-1+ cells were significantly increased after limb ischemia and showed a peak value at day 14 (≈7-fold versus the preischemic value) (from 0.01±0.002% to 0.073±0.002%, $P<0.001$, n=7). Interestingly, such a Lin−/Flk-1+ population was further increased by CO_2 bathing and showed a peak value at day 14 (≈24-fold increase, from 0.01±0.002% to 0.24±0.03%, $P<0.001$, n=7) (Figure 7A). The Lin−/Flk-1+ cells mobilized by CO_2 bathing were mostly positive for anti-endoglin antibody and in the CD34-negative fraction (Figure 7B). Considering that CD34−/Flk-1+ cells rather than CD34+/Flk-1+ are reported to be a real population of hematopoietic stem cells,[17] our present data

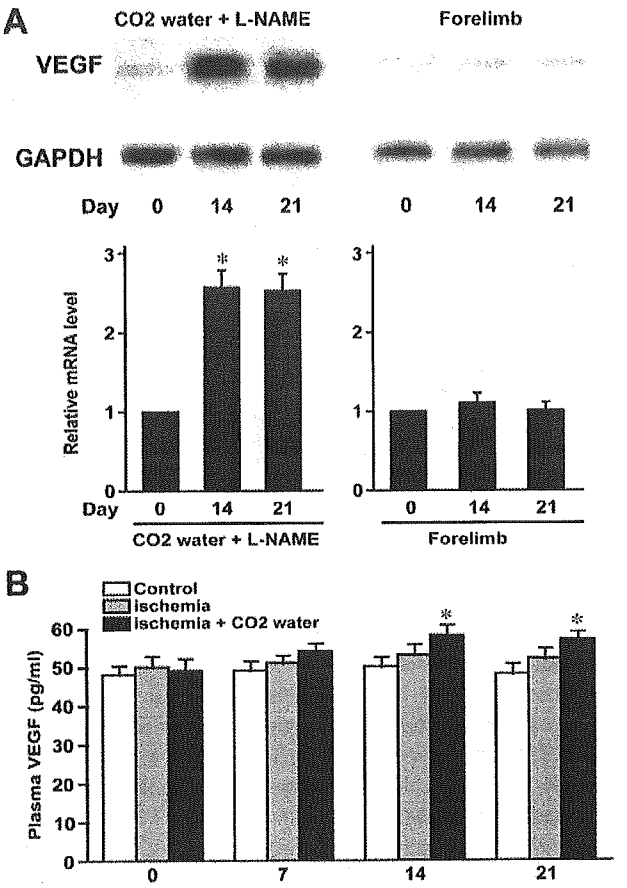

Figure 5. Effect of L-NAME on VEGF mRNA expression in hindlimb and forelimb skeletal muscles after hindlimb ischemia and plasma VEGF concentrations. A, Mice with hindlimb ischemia were provided water containing 1 mg/mL L-NAME, and time-dependent VEGF mRNA expression was measured as described in Figure 4. Forelimb skeletal muscles were dissected from hindlimb ischemia mice, and VEGF mRNA was analyzed. Results (mean±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_2 bathing of normal mice; ischemia, freshwater-bathed ischemic mice; ischemia+CO_2 water, CO_2-bathed ischemic mice, n=5 each). *$P<0.05$ vs day 0.

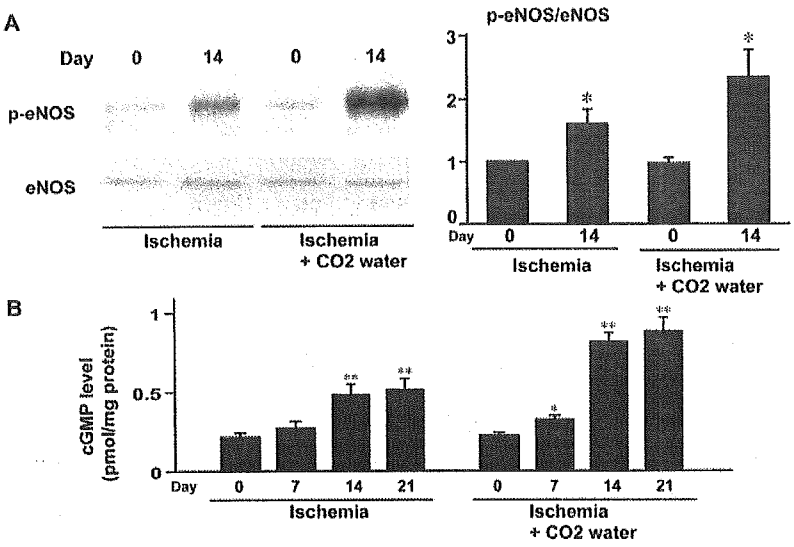

Figure 6. cGMP accumulation in ischemic limbs. A, Skeletal muscles were homogenized and immunoblotted with anti-phospho antibodies for eNOS. Phospho signals in filters were stripped and reprobed with anti-eNOS antibody. Phospho-eNOS densities were measured by densitometry and normalized relative to those of eNOS signals. Results are arbitrarily indicated as values relative to signal densities in day 0 preischemic control. Results shown are mean±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±SEM (n=6 each). *$P<0.05$, **$P<0.001$ vs day 0 preischemic control.

indicate that CO_2-enriched water bathing mobilizes very immature hematopoietic stem cells, including endothelial progenitor cells. To prove the specificity of anti-CD34 antibody, CD34+ 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−/Flk-1+ cells express another endothelial marker, VE-cadherin, and also possess the characteristics of endothelial cells, such as acetylated LDL uptake. The Lin−/Flk-1+ cell population, mobilized by CO_2 bathing, was isolated and cultured with 100 nmol/L VEGF-supplemented medium for 4 days. Approximately 26±1.2% (n=12) of the Lin−/Flk-1+ cells adhered onto the fibronectin-coated plastic dishes. Approximately 74±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_2-enriched water on subcutaneous microcirculation have been published. Savin et al[18] reported that transfer of CO_2 across the skin can have beneficial local vasomotor effects. Hartmann et al[19] demonstrated an increase in tissue oxygen brought about by the Bohr effect in addition to the vasodilation effect by CO_2 or vasodilation by decrease in plasma catecholamine levels. Toriyama et al[20] also reported that the effect of CO_2-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[4] and in isolated aortic strips[5] have suggested that vasodilation in response to CO_2 may be mediated in part by NO. Consistent with these previous studies, our present study demonstrates that immersion of ischemic hindlimb into CO_2-enriched water bathing causes an NO-dependent increase in collateral blood perfusion, induction of regional VEGF synthesis, and mobilization of endothelial-lineage progenitor cells into the circulation.

What is the mechanism responsible for the proangiogenic effect by CO_2-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.[12] VEGF was well known to mobilize endothelial progenitor cells from bone marrow into the circulation.[21] Our present data clearly indicate that VEGF expression is markedly induced in hindlimb skeletal muscles after CO_2-enriched water bathing. A high concentration of CO_2 (1000 to 1200 mg/L) liberates free CO_2 in the freshwater (pH 7.0), resulting in a decrease in pH level (pH 5.0). We found that CO_2 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,[12] the local tissue acidosis by CO_2 bathing, rather than the CO_2 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[22,23] and promotes the recovery of disturbed endothelium-dependent flow in the ischemic hindlimb.[24] Involvement of NO in the angiogenic properties of VEGF has been established in the NO-deficient mice; Murohara et al[10] showed NO-mediated angiogenesis in the hindlimb ischemia model, and Aicher et al[25] 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_2 bathing without affecting local VEGF synthesis. Taken together, these findings demonstrate that the proangiogenic effect by CO_2 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

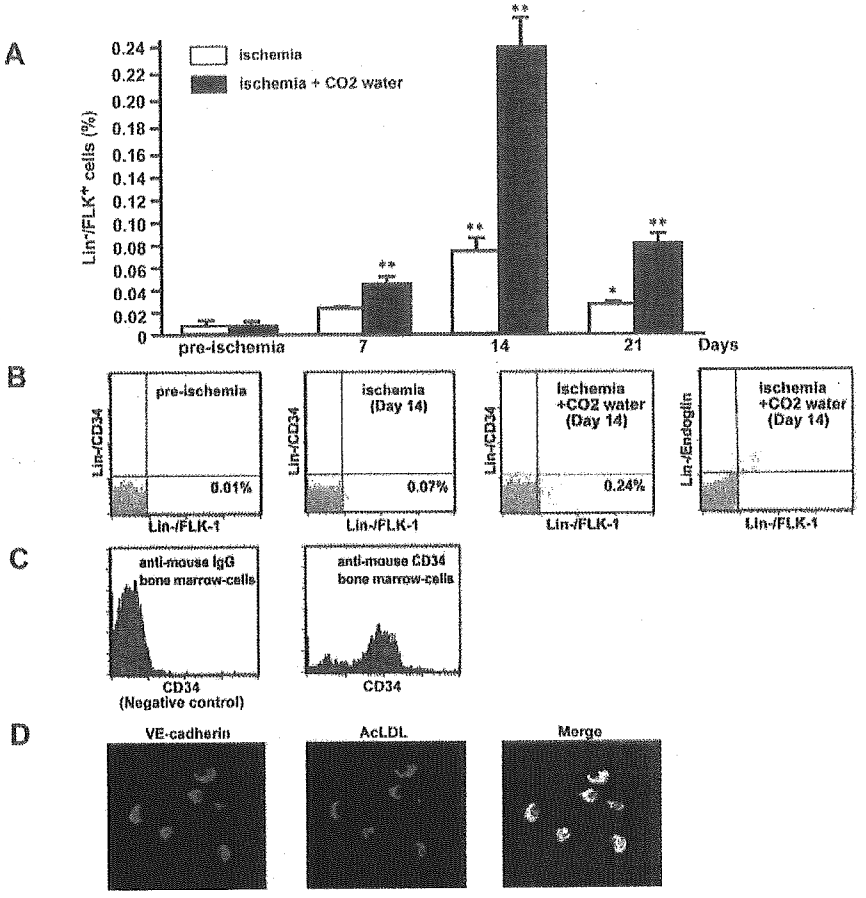

Figure 7. FACS analysis of circulating endothelial-lineage progenitor cells. A and B, Lin−/Flk+ cells in peripheral blood nuclear cells were isolated by PE-labeled lineage antigens, FITC-CD34 and biotin–Flk-1, and then analyzed. Lin−/Flk+/endoglin+ cells were isolated by FITC-labeled lineage antigens, PE-Flk-1 and biotin-endoglin. Cell number ratio of Lin−/Flk+ 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+ 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−/Flk+ cells were cultured on fibronectin-coated plastic dishes in DMEM supplemented with 100 ng/mL VEGF and 10% FCS. After 4 days, DiI-labeled acetylated LDL 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.

progenitor cells is NO-dependent,[25] our present study suggests that CO_2 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[19] reported that repeated CO_2-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[20] also showed that CO_2 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_2-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_2-enriched water bathing therapy can be included in angiogenic therapies associated with neovascularization, such as the transplantation of bone marrow mononuclear cells[14] or VEGF gene therapy.[26]

Acknowledgments

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Original Article

Mechanism for IL-1β-mediated neovascularization unmasked by IL-1β knock-out mice

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Abstract

We have reported that interleukin-1 beta (IL-1β) upregulates cardiac expression of vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR-2), raising the possibility that IL-1β plays an important role in VEGF-mediated neovascularization. In this study, we examined the cellular mechanism for ischemia-induced neovascularization using IL-1β knock-out (–/–) mice. Recovery of blood perfusion in ischemic hindlimb in IL-1β–/– mice was markedly (43% decrease) impaired as compared with the wild-type mice. $CD31^+$ vessel numbers and $Ki\text{-}67^+$ neo-capillaries were significantly ($P < 0.01$) decreased 44% and 68%, respectively. IL-1β expression was localized in the capillary vessels in ischemic limb muscles. Ischemia-induced expressions of hypoxia-inducible factor 1 alpha (HIF-1α), VEGF, its receptor VEGFR-2 and vascular cell adhesion molecule-1 (VCAM-1) were markedly inhibited in the IL-1β–/– mice. Hindlimb ischemia-induced an increase (1.22% out of total nuclear cell) in $CD34^-/B220^-/CD3^-/Flk\text{-}1^+$ hematopoietic stem cell population in peripheral blood in the wild-type mice, whereas in the IL-1β–/– mice such increase was only 0.09%. Injection of IL-1β protein into the wild-type mice markedly increased the ratio of the $CD34^-/B220^-/CD3^-/Flk\text{-}1^+$ cell population (from 0.03% to 0.7%) in the peripheral blood associated with an increase in the number of endothelial cells. Such IL-1β-mediated increases in cell numbers were blocked by co-injection of anti-VEGF antibody. $CD34^-/B220^-CD3^-Flk\text{-}1^+$ cells trans-differentiated into eNOS- and CD31-expressing endothelial cells in vivo and in vitro. This study demonstrates that IL-1β plays a key role in ischemia-induced neovascularization by mobilizing $CD34^-/B220^-CD3^-Flk\text{-}1^+$ endothelial precursor cells in a VEGF-dependent manner as well as by upregulating expressions of VEGF, VEGFR-2 and adhesion molecules on endothelial cells.

Keywords: IL-1β; HIF-1α; VEGF; Ischemia; Neovascularization; Angiogenesis

1. Introduction

Neo-vascularization plays critical role in versatile physiological and pathological aspects. Understanding the molecular mechanisms of neovascularization is essential to develop effective therapy for many diseases. There are two systems of neovascularization, angiogenesis and vasculogenesis [1]. Vasculogenesis had been thought to occur only in the development of the vascular system of the fetus. Endothelial precursor cell (EPC) have been found to mobilize into adult peripheral blood in response to regional ischemia and play a critical role in neovascularization in the ischemic region [2]. EPC is also shown to be mobilized by vascular endothelial growth factor (VEGF) from bone marrow [3].

Analysis using knock-out mice revealed that each angiogenic growth factor plays distinctive roles in neovascularization. VEGF plays a critical role in primary vessel network formation by the upregulation of endothelial cell growth and survival [4]. There are three kinds of VEGF receptors (VEGFRs), Flt-1 (VEGFR-1), Flk-1 (VEGFR-2) and neuropilin. VEGFR-2 knock-out mice exhibited embryonic death due to the impaired development of endothelial and hematopoietic cells [5]. VEGFR-1 knock-out mice showed embryonic death due to mass of endothelial cells caused by their over-proliferation in the vascular lumen [6]. Further, neuropilin has been shown to enhance VEGF165 binding to the Flk-1 receptor [7]. Actually, neuropilin knock-out mice exhibited

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embryonic death due to the impaired development of endothelial cells, similar to the phenotype of Flk-1 knock-out mice [8]. Besides the development of the vascular system in the fetus, VEGF in adults plays critical roles in placental formation and wound healing as well as tumor growth and neovessel formation in diabetic nephropathy. In contrast, angiopoietins are involved in the trimming and reinforcement of the primary vessel network with the stabilization of endothelial cells by recruiting pericytes [9,10]. Ehprin-B2 and its receptor Eph-4 play an important role in molecular distinction and angiogenic interaction between arteries and veins.

We have reported that interleukin-1 beta (IL-1β) induces the expression of VEGF in cardiac endothelial cells [11]. Other pro-inflammatory cytokines, including IL-6 [12], IL-8 [13], IL-18 [14] and tumor necrotic factor alpha (TNF-α) [15], have been shown to control the neovascularization process. The administration of IL-1β-induced intense neovascularization in vivo [16]. IL-1β-induced angiogenesis is mediated by upregulating the expression of VEGF in inflammatory cells [17,18], especially under ischemic conditions [19], although IL-1β itself does not have angiogenic activity. IL-1β-induced VEGF expression is mediated by the activation of hypoxia-inducible factor 1 alpha (HIF-1α), the p38 mitogen-activated protein kinase (MAPK) pathway [20] and the activation of protein kinase C. However, the involvement of endothelial progenitor cells mobilized by IL-1β in response to ischemia remains to be fully clarified. To address this question, we analyzed neovascularization in an ischemic hindlimb model using IL-1β knock-out mice [21].

Here, we confirmed the functional role of IL-1β in neovascularization. The present study demonstrated that neovascularization in response to ischemia was markedly reduced in IL-1β knock-out mice. The expression of both IL-1β and VEGF was localized in CD31-positive vessels in ischemic limbs, and absence of IL-1β caused impairment of VEGF-induced mobilization of endothelial progenitor cells into the peripheral blood.

2. Materials and methods

2.1. Materials

IL-1β–/– mice were obtained from Dr. Sudo and Dr. Iwakura [21]. Green fluorescence protein (GFP) transgenic mice were generously obtained from Dr. Okabe [22].

2.2. IL-1β–/– mice and hindlimb ischemia

IL-1β–/– and littermate male control mice (C57BL/6J) at 8–12 weeks old were used. Unilateral hindlimb ischemia was induced by ligation and cutting the left femoral artery. One to 3 weeks after operation, skeletal muscles were removed and snap-frozen in liquid nitrogen. Animal protocol was approved by the Institutional Animal Care and Use Committee.

2.3. Laser Doppler perfusion image

We measured the ratio of the ischemic (left)/normal (right) limb blood flow using a laser Doppler perfusion image (LPDI) analyzer (Moor Instruments, Devon, UK). After scanning blood flow twice, stored images were subjected to computer-assisted quantification of blood flow, and the average flows of ischemic and non-ischemic limbs were calculated. To minimize data variables due to ambient light and temperature, the LPDI index was expressed as the ratio of ischemic (left) to non-ischemic (right) limb blood perfusion.

2.4. FACS staining

Seven days after operation of ischemic limb model, retro-orbital blood was collected immediately before sacrifice. Total nuclear cell in peripheral blood were isolated by erythrocyte lysis with ammonium chloride solution (PharM Lyse, Bector Dickinson), stained with FITC-labeled anti-CD34 or anti-CD49d (α4 integrin), PE-labeled anti-Flk-1 as well as Cy5-labeled anti-CD3 and anti-B220 antibodies (Pharmingen) and analyzed using a FACScan™ flow cytometer.

2.5. Immunohistochemical analysis

Four pieces of ischemic tissues from the adductor and semi-membranous muscles were removed and snap-frozen by liquid nitrogen. Five micrometers frozen sections were stained with anti-PE-labeled CD31 antibody, anti-FITC-labeled CD34 or vascular cell adhesion molecule-1 (VCAM-1) antibody (Pharmingen) as endothelial cell marker, also anti-IL-1β antibody (Santa Cruz) with anti-FITC-conjugated rabbit antibody (Vector). Specimens were observed with Olympus Fluoview Confocal Microscope and images were analyzed with Adobe Photoshop soft program. Also, other specimens were blocked endogenous peroxidase activity by incubation with 3% hydrogen peroxidase. Subsequently, they were incubated with anti-Ki-67 antibody (Dako), Bound primary antibody was detected using the avidin–biotin–immunoperoxidase method (Dako) and subsequently visualized by DAB (Vector). Twenty fields from two muscle samples were randomly selected for statistical evaluation.

2.6. Western blot analysis

In order to determine the amount of VEGF, VEGFR-2 and HIF-1α in the ischemic limb muscle of IL-1β–/– and wild-type mice, lysates from ischemic limb muscle were subjected to western blot using anti-VEGF, anti-VEGFR-2 and anti-HIF-1α antibody. These antibodies were visualized with an enhanced chemiluminescence system (Amersham).

2.7. Reverse transcription-polymerase chain reaction to test the expression of IL-1β

The expression of IL-1β in ischemic tissue was evaluated by reverse transcription-polymerase chain reaction (RT-

PCR). Samples from the adductor and semi-membranous muscles at 1 d after or before operation for hindlimb ischemia were frozen and homogenized in TRIZOL Reagent (GIBCO BRL) and total RNA was extracted. Then, RT-PCR was performed on the tissue samples with mouse IL-1β primer set kit (Maxim Biotech. Inc.). The PCR products were compared with the simultaneously amplified product of the β-actin for calibration of equal amount of mRNA.

2.8. Differentiation of Flk-1$^+$ cell into endothelial cells in vivo

The GFP$^+$CD3$^-$B220$^-$Flk-1$^+$ cells were isolated by Fluorescence Cell Sorter from the peripheral blood of enhanced green fluorescence protein (EGFP) transgenic mice (C57BL/6J strain) that had undergone operation for hindlimb ischemia 3 d before. Then, 2×10^6 cells were intravenously transplanted into the same strain mice that also had been treated with operation for hindlimb muscle ischemia prior to 1 d before transplantation. Seven days after transplantation, ischemic hindlimb muscle or non-treated limb muscle were removed and observed the presence of EGFP-positive cells with immunostaining by PE-labeled anti-CD31 antibody.

2.9. Differentiation of Flk-1$^+$ cell into endothelial cells in vitro

The population of CD3$^-$B220$^-$Flk-1$^+$cells was isolated with Fluorescence Cell Sorter from the peripheral blood of the mice that had undergone limb ischemic operation 3 d before. Then, these cells were primary cultured on fibronectin-coated plastic dishes in DMEM supplemented with 100 ng/ml VEGF and 10% fetal bovine serum. After 10 d, DiI-labeled acetylated LDL (Biomedical Technologies Inc.) were added into medium at 2 μg/ml for 6 h and subsequently fixed with 4% paraformaldehyde, stained with anti-eNOS antibody (Santa Cruz) with FITC-labeled anti-rabbit IgG antibody (Vector).

2.10. Statistics

Statistical analyses were performed by one-way ANOVA followed by pairwise contrasts using Dunnett's test. Data (mean ± S.E.) were considered significant when P was <0.05.

3. Results

3.1. Impaired recovery of blood perfusion in ischemic hindlimb of IL-1β–/–mice

As we previously reported that IL-1β induces expression of VEGF and its receptor [11], we assumed that IL-1β promotes neovascularization via induction of VEGF. We examined the blood flow recovery in ischemic hindlimb of IL-1β–/– mice by a LPDI analyzer. Three weeks after the operation for hindlimb ischemia, the recovery of blood flow in the IL-1β–/– mice was significantly decreased (43%, $P < 0.01$) than that in the wild-type mice (Fig. 1A, also quantitative analysis in Fig. 1B). Replacement of 0.5 μg of IL-1β protein into ischemic muscle of IL-1β–/– mice at 0, 2, 4 and 6 d after operation normalized the decreased blood flow toward the wild-type level (Fig. 1B).

3.2. Decrease in vessel numbers in ischemic hindlimb muscle of IL-1β–/– mice

We examined total vessel numbers and neo-capillary formation by analyzing CD31- and Ki-67-positive cells. No significant difference was observed in the basal vessel numbers in the hindlimb muscle between the wild-type and IL-1β–/– mice. Three weeks after ischemia, CD31-positive vessel numbers were significantly increased in the wild-type mice (1.6 ± 0.2 to 2.9 ± 0.3 cells/muscle fiber, $P < 0.01$, $n = 12$) (Fig. 2A). Ki-67-positive neo-capillary formation was also enhanced in the wild-type mice (0.7 ± 0.1 to 2.2 ± 0.3 cells/muscle fiber, $P < 0.01$, $n = 12$) (Fig. 2B). In contrast, CD31-positive vessel numbers and neo-capillary formation in response to ischemia did not increase in the IL-1β–/– mice (CD31: 1.5 ± 0.1 to 1.5 ± 0.2; Ki-67: 0.6 ± 0.1 to 0.7 ± 0.1) (Fig. 2A,B).

3.3. Localization of IL-1β in ischemic hindlimbs and reduced induction of VEGF system

IL-1β mRNA was substantially induced in ischemic hindlimb muscle at 1 d after operation in wild-type mice, whereas no mRNA message was detected by RT-PCR in IL-1β–/– mice (Fig. 3A). Localization of induced IL-1β corresponded to CD31-positive capillaries in ischemic hindlimbs and such induction was not observed in IL-1β–/– mice (Fig. 3B). As we previously demonstrated the relationship between IL-1β and VEGF system [11], we next examined the induction of VEGF, VEGFR and HIF-1α. Western blotting showed ischemia-mediated rapid induction of HIF-1α, VEGF and VEGFR-2 in the wild-type mice; they were markedly up-regulated as early as 1 d after hindlimb ischemia, reached a peak level at days 1–3 and thereafter declined, whereas such induction in VEGF system was abolished in the IL-1β–/– mice, suggesting that induction of IL-1β in hindlimb ischemia plays a central role in the expressions of HIF-1α, VEGF and VEGFR (Fig. 3C).

3.4. IL-1β mobilized EPCs into peripheral blood by VEGF-mediated mechanism

CD34$^+$AC133$^+$/Flk-1$^+$ hematopoietic stem cells circulate in the peripheral blood of human as an EPC and play a critical

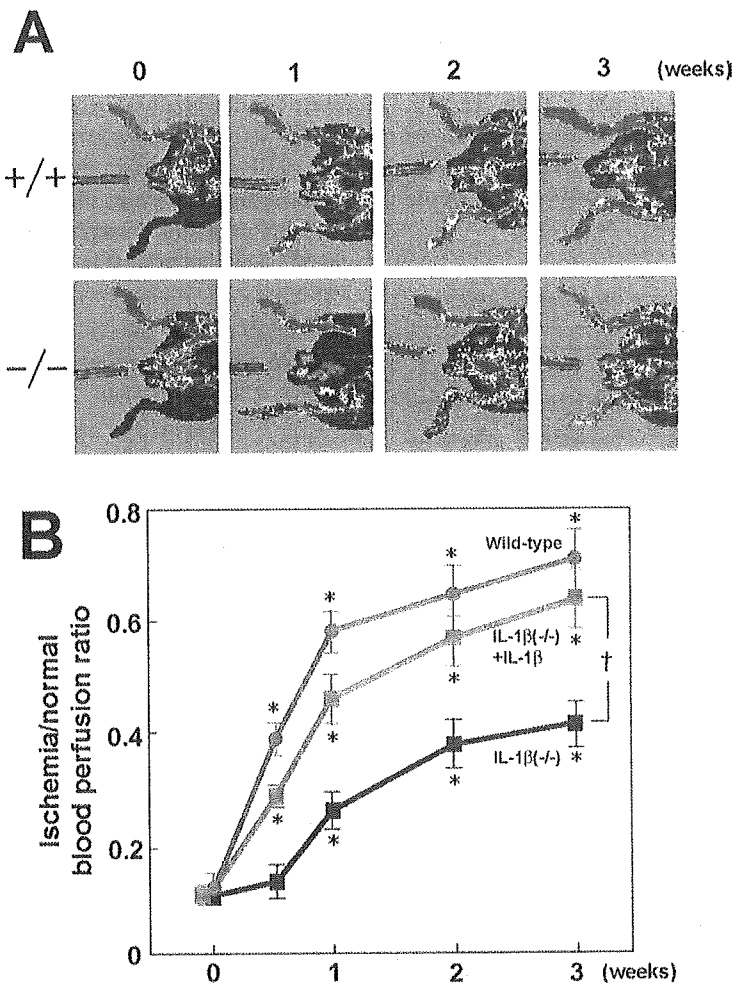


Fig. 1. (A) Time course recovery of the blood flow in the ischemic limbs of IL-1β–/– and wild-type mice, observed with a LPDI analyzer. The blue colored area indicates a low blood supply region. The red colored area indicates a high blood supply region. After operation for limb muscle ischemia, the recovery of blood flow in the ischemic limb of the IL-1β–/– mice was significantly reduced. (B) Decrease in the blood flow recovery of the ischemic hindlimb of the IL-1β–/– mice, calculated as the ratio of the ischemic (left)/normal (right) limb blood flow. Green line and black line indicate the relative blood flow in the ischemic limbs of the wild-type and IL-1β–/– mice, respectively. * $P < 0.01$ between IL-1β–/– and wild-type control mice. Also, four times intra-muscular injection of 0.5 μg IL-1β into the ischemic muscle of the IL-1β–/– mice at days 0, 2, 4 and 6 improved their blood flow recovery (red line). * $P < 0.01$ between IL-1β-injected mice and non-injected IL-1β–/– mice (†).

role in neovascularization in ischemic tissue [2]. As VEGF was shown to mobilize this cell population [3], we analyzed whether lack of IL-1β affects such cell population in the peripheral blood. As AC133 is not available for mouse, we gated $CD3^-B220^-$ (T and B cell marker) cells as a lymphoid negative fraction and then analyzed CD34 and Flk-1 population. FACS analysis indicated that $CD3^-B220^-/Flk\text{-}1^+$ cell population is barely detected in the peripheral blood of both wild-type (0.09 ± 0.006% of total nuclear cells) and IL-1β–/– (0.03 ± 0.002%) mice. Seven days after limb ischemia, $CD3^-B220^-/Flk\text{-}1^+$ cell population in the peripheral blood of wild-type mice was markedly increased ~14-fold (from 0.09% to 1.22%) (Fig. 4, upper panel). Interestingly, the increased $CD3^-B220^-/Flk\text{-}1^+$ cells were mostly CD34-negative fraction. Considering that $CD34^-Flk\text{-}1^+$ cells rather than $CD34^+Flk\text{-}1^+$ are reported to be a real population of hematopoietic stem cells [23], our present data may suggest that hindlimb ischemia mobilizes a very immature hematopoietic stem cells including endothelial-lineage cells. In contrast, such ischemia-mediated increase in $CD3^-B220^-/Flk\text{-}1^+/CD34$ population was only ~3-fold (from 0.03% to 0.09%) in the IL-1β–/– mice (Fig. 4, lower panel). Thus, mobilization of $CD3^-B220^-Flk\text{-}1^+$ cell population into the peripheral blood in response to limb ischemia was defective in the IL-1β–/– mice.

We next studied whether addition of IL-1β can reverse the impairment of capillary formation or mobilization of $CD3^-B220^-Flk\text{-}1^+$ cell population in the IL-1β–/– mice. As shown in Fig. 5A,B, four times injection of 0.5 μg of IL-1β at every 2 d into hindlimb muscle of IL-1β–/– mice induced increase in the number of $CD31^+$ endothelial cells in the injected muscle as well as $CD3^-B220^-Flk\text{-}1^+$ cell population in the peripheral blood. The extents of which were comparable to ischemia-mediated increase in the wild-type mice. Interest-

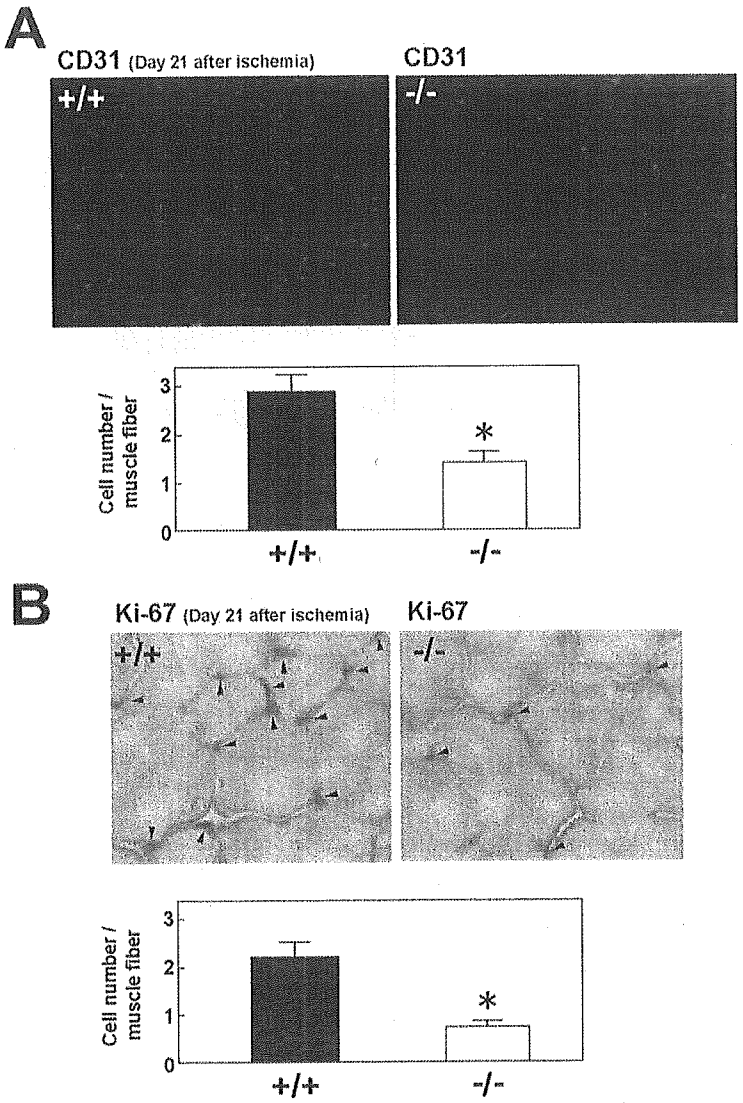


Fig. 2. (A) Measurement of the number of endothelial cells. In the upper panel, the red dots indicate the endothelial cells stained with an anti-CD31 antibody. The lower panel shows the number of endothelial cells in a low magnified microscopic field. Statistically, the number of endothelial cells in the IL-1β–/– mice was less than the wild type. * $P < 0.05$. (B) Measurement of the number of proliferating cells. In the upper panel, the brown dots (black arrowhead) indicate the proliferating cells, stained with an anti-Ki-67 antibody. The lower panel shows the quantification of the number of proliferating (Ki-67-positive) cells per low magnified microscopic field. Statistically, the number of proliferating cells in the IL-1β–/– mice was less than in the wild type. * $P < 0.05$.

ingly, four times co-administration of 10 μg of anti-VEGF antibody completely blocked IL-1β-mediated capillary formation (Fig. 5A,C) and mobilization of $CD3^-B220^-Flk\text{-}1^+$ cell population (Fig. 5B,C). Taken together, these findings suggest that IL-1β-mediated vasculogenesis is mainly mediated by VEGF-dependent manner.

IL-1β was shown to promote expressions of adhesion molecules on the endothelial cell [24], which are important for homing of endothelial precursors. We therefore examined the induction of VCAM-1 in ischemic muscle and the expression of its binding molecules, integrin α4β1 or α4β7 [25,26], on $CD3^-B220^-Flk\text{-}1^+$ cells. VCAM-1 was significantly induced 1 d after limb ischemia in the wild-type mice ($P < 0.01$, 3.4 ± 0.3-fold vs. 0 time control, $n = 4$), while its induction in the IL-1β–/– mice was much weaker (1.8 ± 0.3-fold vs. 0 time control, $n = 4$) (Fig. 6A, left panel). VCAM-1 expression was detected on $CD31^+$ endothelial cells (Fig. 6A, right panel). FACS analysis indicated that the majority (86 ± 5%, $n = 4$) of the $CD3^-B220^-Flk\text{-}1^+$ cells expressed α4 integrin (Fig. 6B). These data suggest that VCAM-1 expression was induced by IL-1β and $CD3^-B220^-/Flk\text{-}1^+$ cells are mobilized into the ischemic region through the interaction of α4 integrin with VCAM-1 on endothelial cells.

3.5. The $CD3^-B220^-Flk\text{-}1^+$ cell population contains EPC

We tested whether the $CD3^-B220^-Flk\text{-}1^+$ cell population actually contains the cells that are capable of differentiating into functional endothelial cells. The $CD3^-B220^-Flk\text{-}1^+$ cell population in the peripheral blood was isolated by FACS

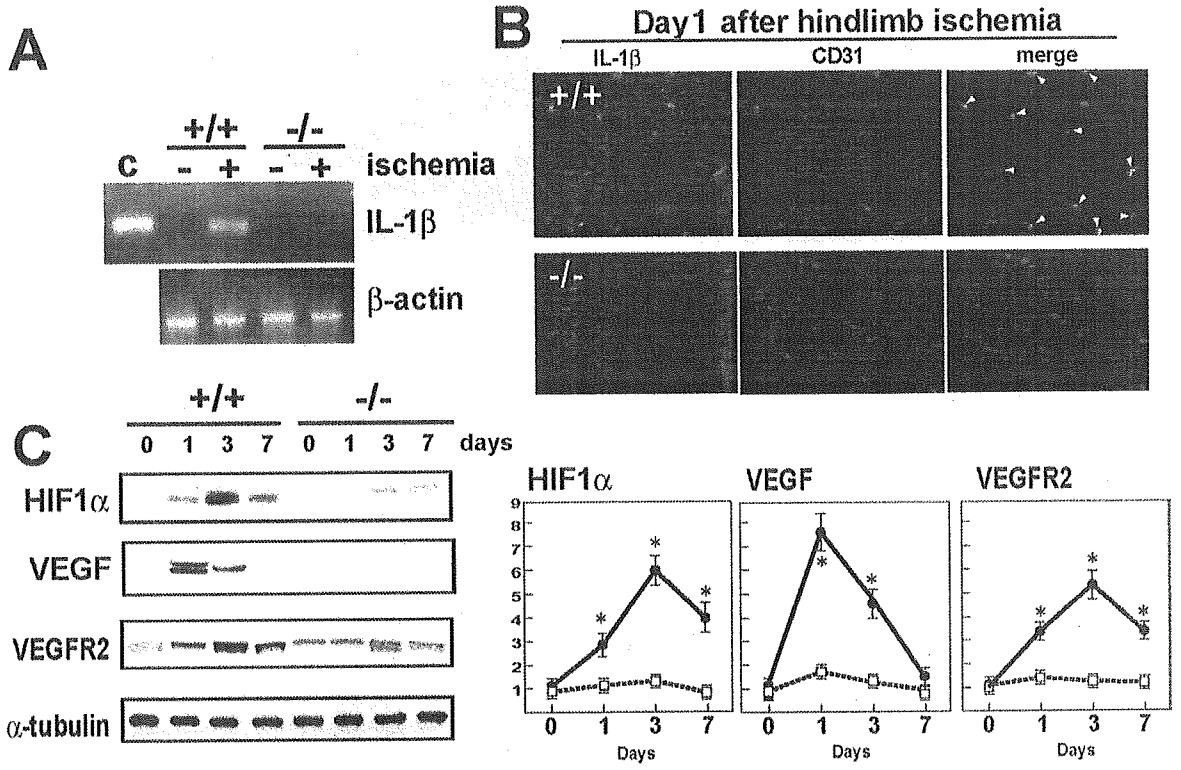


Fig. 3. Expression of IL-1β in ischemic limbs. (A) Detection of IL-1β and β-actin as calibration markers of equal amounts of total RNA, with RT-PCR method in the ischemic hindlimb muscle 1 d after the operation. (B) Immunohistochemical analysis of the distribution of IL-1β in ischemic limb muscle 1 d after operation. The majority of the cells expressing IL-1β (green spots) were CD31 positive (red spots). Cells positive for both markers are observed as yellow dot in the merged picture (white arrow). (C) Impaired expression of VEGF, VEGFR-2 and HIF-1α in ischemic limb muscle with western blot analysis. Total tissue lysates containing equal amounts of protein were subjected to western blot with anti-VEGF, anti-VEGFR-2, anti-HIF-1α and also anti-α-tubulin antibody, respectively. Expression of VEGF, VEGFR-2 as well as HIF-1α in the ischemic muscle of the IL-1β–/– mice was significantly less than in that of the wild type. α-Tubulin is the calibration markers for equal amounts of protein. The right three panels at (C) show the time course of the relative intensity of VEGF, VEGFR-2 as well as HIF-1α bands in the western blot analysis, measured by the Adobe Photoshop soft program. * $P < 0.005$ vs. IL-1β knock-out mice.

from mice that had already been undergone limb ischemic operation 3 d before, and then cultured with 100 nM VEGF supplemented medium for 10 d. Approximately, 10 ± 1.4% (n = 12) of the plated cells adhered onto the fibronectin-coated plastic dishes and expanded. Differentiation of endothelial-like cells was examined by the expression of eNOS and ability to incorporate acetylated LDL. We observed that 51 ± 2.3% (n = 12) of the growing cells showed the ability of incorporating the DiI-labeled acetylated LDL and that these cells expressed the eNOS (Fig. 7A). We further studied whether other fractions, such as $CD34^-/B220^-CD3^-/Flk\text{-}1^-$ fraction and $B220^+CD3^+$ fraction, were capable of differentiating into endothelial cell. We primarily cultured these cells in VEGF supplemented medium. Most of $B220^+CD3^+$ cells did not adhere onto the culture dish. Although ~30% of $CD34^-/B220^-CD3^-/Flk\text{-}1^-$ cells attached onto tissue culture dish, neither eNOS expression nor incorporation of DiI-labeled acetylated LDL was observed in these attaching cells (data not shown). Taken together, these data suggested that the $CD3^-B220^-Flk\text{-}1^+$ cell population includes the endothelial progenitor cells that can differentiate into functional endothelial cells, and that other cell populations unlikely contain the endothelial precursors.

We also examined whether the $CD3^-B220^-Flk\text{-}1^+$ cells contribute to neo-capillary formation in ischemic hindlimb. We isolated the $CD3^-B220^-Flk\text{-}1^+$ cell population expressing the EGFP from the peripheral blood of EGFP (C57BL/6J strain) transgenic mice 3 d after operation for hindlimb muscle ischemia. Subsequently, we intravenously transplanted 2×10^6 cells into the same strain mice that had already been operated for hindlimb ischemia 1 d before transplantation. Seven days after cell transplantation, the hindlimb muscle was removed and the presence of GFP-positive cells was examined. Fig. 7B shows that GFP-positive cells were, indeed, detected in the hindlimb muscle and these GFP-positive cells were also positive for the endothelial cell marker, CD31 (Fig. 7B, upper panel). On the contrary, the GFP-positive cells were barely detectable in non-treated muscle (Fig. 7B, lower panel), suggesting that $CD3^-B220^-Flk\text{-}1^+$ cells contain the cell population that can differentiate into CD31-positive endothelial cells in the ischemic muscle.

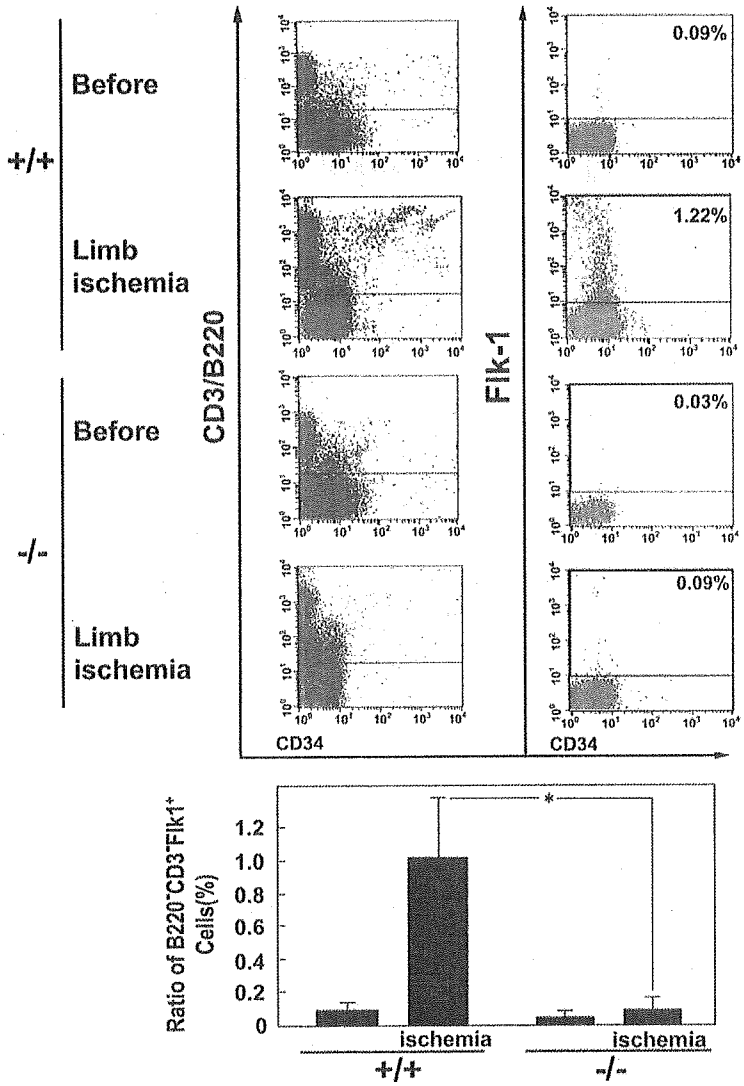
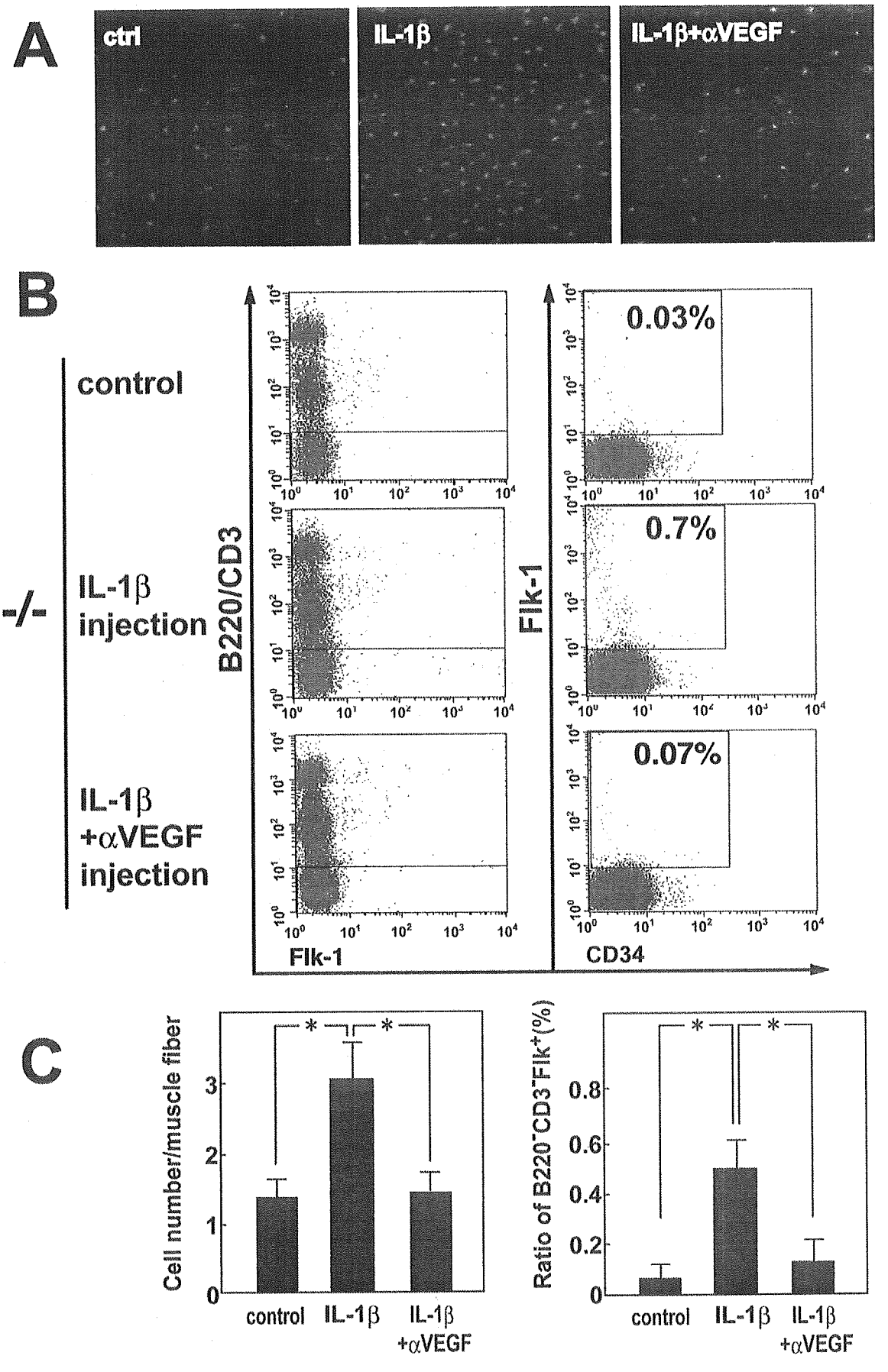


Fig. 4. FACS analysis for the $CD3^-B220^-Flk\text{-}1^+$ cells in the total nuclear cells of the peripheral blood in the wild-type or IL-1β–/– mice before and 7 d after treatment with limb ischemic surgery. Isolated nuclear cells in the peripheral blood were subjected to FACS analysis with anti-CD3, anti-B220, anti-CD34 and also anti-Flk-1 antibodies. The cell number ratio of $CD3^-B220^-Flk\text{-}1^+$ cells to the total nuclear cells is shown in the Figure. In wild-type mice, it increased from 0.09% to 1.22% after surgery. In IL-1β–/– mice, it increased from 0.03% to 0.09% after surgery. Experiment was repeated for three times with similar results. This figure of FACS analysis is the representative one. Statistical analysis is shown at lower panel. * $P < 0.005$ between the ratio of $CD3^-B220^-Flk\text{-}1^+$ cell number in the wild-type mice and IL-1β–/– mice after limb ischemia.

4. Discussion

Besides versatile angiogenic growth factors such as VEGF, angiogenic action by inflammatory cytokines has been poorly defined. Voronov et al. [27] have very recently reported that tumor angiogenesis is inhibited in the IL-1β–/– mice, whereas it remains to be determined whether IL-1β affects the expression of VEGF or VEGF enhances neovascularization by mobilizing endothelial progenitor cells. We focused on collateral vessel formation in the ischemic hindlimb of IL-1β–/– mice, and demonstrated that (1) IL-1β enhanced neo-capillary formation in the ischemic hindlimb in a VEGF-dependent manner, (2) IL-1β upregulated the expressions of HIF-1α, VEGF, its receptor and adhesion molecule VCAM-1, (3) IL-1β mobilizes $CD34^-Flk\text{-}1^+$ cells rather than $CD34^+Flk\text{-}1^+$ cells as an endothelial progenitor cells by a VEGF-dependent mechanism, which in turn contributes to neo-capillary formation, and that (4) the isolated $CD34^-Flk+$ cells trans-differentiate to functional endothelial cells in culture with VEGF. Considering that highly undifferentiated hematopoietic stem cells were currently shown to be CD34 negative [23], this study presents a novel interesting finding that ischemia or IL-1β mobilizes a rather undifferentiated $CD34^-Flk\text{-}1^+$ hematopoietic stem cells including endothelial-lineage cells.

What is the essential role of IL-1β in neovascularization, based on the analysis of IL-1β–/– mice? In physiological condition, the fluid shear stress activates eNOS, leading to


A
ctrl
IL-1β
IL-1β+αVEGF
B
-/-
control
IL-1β injection
IL-1β +αVEGF injection
B220/CD3
Flk-1
0.03%
0.7%
0.07%
CD34
C
Cell number/muscle fiber
control
IL-1β
IL-1β +αVEGF
Ratio of B220⁻CD3⁻Flk⁺(%)

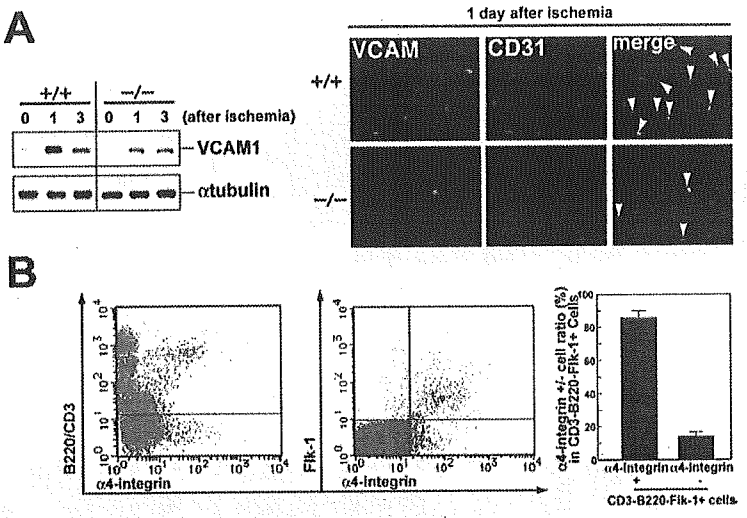


Fig. 6. Induction of VCAM-1 in the endothelial cells of ischemic muscle and α4 integrin expression on CD3⁻B220⁻Flk-1⁺ cell. (A) At 1 and 3 d after hindlimb ischemia, VCAM-1 expression in ischemic hindlimb muscle of wild-type or IL-1β–/– mice was studied by western blot analysis (left panel). The frozen section of day 1 ischemic muscle was stained with FITC-labeled anti-VCAM-1, PE-labeled anti-CD31 antibody (right panel). (B) Total nuclear cells in peripheral blood were analyzed by FACS 7 d after hindlimb ischemia. The number in the FACS data represents the cell number ratio of the CD3⁻B220⁻/Flk-1⁺/α4 integrin⁺ or the CD3⁻B220⁻/Flk-1⁺/α4 integrin⁻ cell in the total nuclear cell. 86 ± 5% of the CD3⁻B220⁻Flk-1⁺ cells expressed α4 integrin ($n = 4$). Each experiment was repeated for four times with similar results and representative data were shown.

NO production. NO stabilizes endothelial cell function and exerts anti-atherogenic and anti-angiogenetic effect. In contrast, in response to tissue ischemia as well as inflammation, the inflammatory cytokine IL-1 is produced. IL-1 activates endothelial cell, leading to subsequent expression of angiogenic cytokines or chemokines as well as adhesion molecules [24,28] or matrix metalloproteinase (MMP) [29], which provides the environment required for neovascularization. We observed in western blot analysis that the adhesion molecule VCAM-1 was transiently expressed on endothelial cell in the ischemic hindlimb muscle. FACS analysis indicated that the CD3⁻B220⁻Flk-1⁺ cells expressed the VCAM-1 binding molecule, α4 integrin, suggesting that CD3⁻B220⁻Flk-1⁺ cells adhere onto endothelial cells through the association between VCAM-1 and α4 integrin. As the induction of VCAM-1 in ischemic muscles was markedly inhibited in the IL-1β–/– mice, adhesion of EPCs onto endothelial cells also may be impaired. MMP9 is required for sub-endothelial migration of EPCs [29]. We found that the induction of MMP9 is also markedly inhibited in the ischemic muscles of IL-1β–/– mice (Amano et al., unpublished observation). Taken together, IL-1β plays a key role in ischemia-induced neovascularization by mobilizing CD34⁻/B220⁻CD3⁻Flk1⁺ EPCs in a VEGF-dependent manner as well as by upregulating expressions of VEGF, VEGFR-2 and adhesion molecules on endothelial cells.

The presence of lower levels of IL-1β promotes not only the endothelial cell growth but also the direct tissue repairing, such as astroglial growth in an ischemic brain [30]. We observed that muscle fibers in the ischemic limbs of IL-1β–/– mice were more atrophied than in that of the wild type. This phenotypic change may be caused by the lack of a cytoprotective effect of IL-1β. In contrast, in the presence of a high concentration of IL-1β, the harmful effect on ischemic tissue rather occurs. Mizushima et al. [31] showed reduced post-ischemic apoptosis in the hippocampus of IL-1β–/– mice. Touzani et al. [32] also reported that the injection of IL-1β exacerbated ischemic brain damage and post-ischemic apoptosis of the ischemic brain. Thus, the biological effect of IL-1β on ischemic tissue is diverse in each condition.

The IL-1β–/– mice exhibited reduced HIF-1α accumulation, followed by impaired VEGF expression. However, the molecular mechanism by which activation of the IL-1β receptor signaling pathway leads to an increase in the amount of HIF-1α has not been clarified yet. In hypoxic condition, O_2-dependent proline hydroxylation of HIF-1α with prolyl-hydroxylase and subsequent degradation of HIF-1α is inhibited, which eventually result in accumulation of HIF-1α

Fig. 5. IL-1β-induced increase in endothelial cell number of the muscle in IL-1β–/– mice through VEGF. (A) Compared to the pretreated hindlimb muscle (left panel), four times injection of 0.5 μg IL-1β (R&D system) every other day into the hindlimb muscle of the IL-1β–/– mouse, the number of endothelial cells, detected with an anti-CD34 antibody, significantly increased at day 7 (middle panel). In contrast, the four times co-injection of 0.5 μg of IL-1β with 10 μg of anti-VEGF neutralizing antibody (R&D system) abolished increase in the number of endothelial cells induced with IL-1β (right panel). (B) Mobilization of the CD3⁻B220⁻Flk-1⁺ cells into the peripheral blood by IL-1β injection into limb muscle of IL-1β–/– mice. After three times intra-muscular injection of IL-1β–/– mice with 0.5 μg IL-1β every day, the ratio of the CD3⁻B220⁻Flk-1⁺ cell number to the total nuclear cell number at day 3 after ischemia increased from 0.03% to 0.7%. In contrast, three times co-injection of 0.5 μg of IL-1β with 10 μg of anti-VEGF antibody abolished increase in the number of endothelial cells induced with IL-1β (right panel). (C) Statistical analysis of the number of endothelial cells in limb muscle per lower magnified microscopic field (left panel) and also the ratio of CD3⁻B220⁻Flk-1⁺ cell number to the total nuclear cell number in peripheral blood (right panel). * $P < 0.01$.

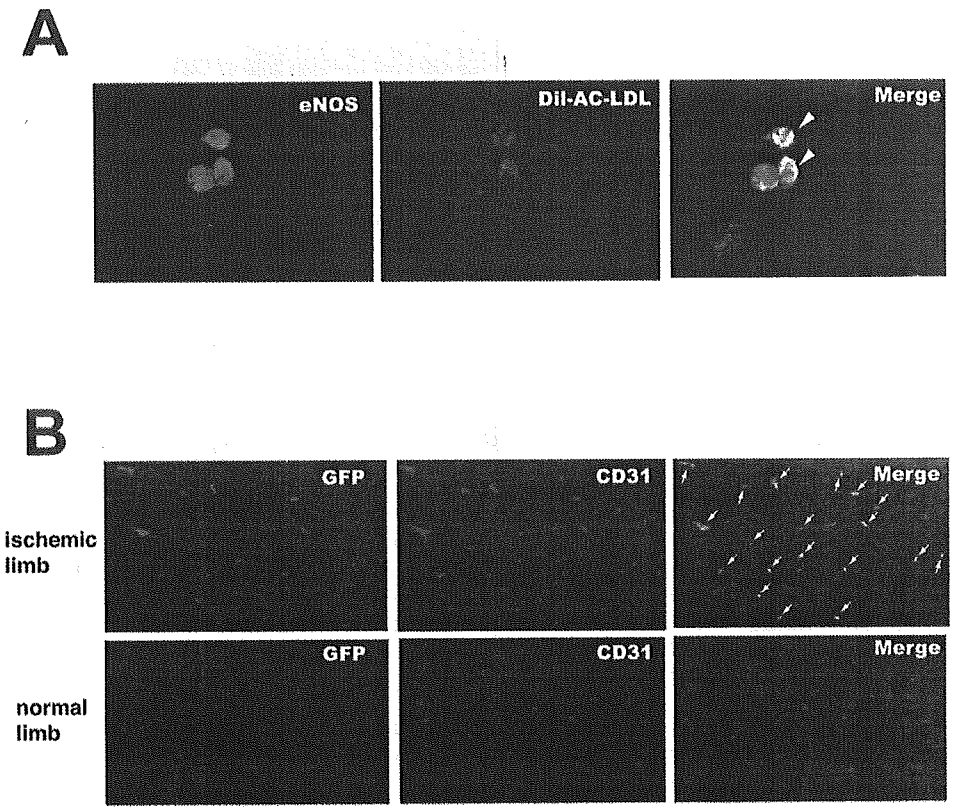


Fig. 7. Characterization of the $CD3^-B220^-Flk\text{-}1^+$ cell population as EPCs. (A) Incorporation of acetylated LDL into the $CD3^-B220^-Flk\text{-}1^+$ cells as characterization for endothelial cells. Three days after limb ischemic surgery, the $CD3^-B220^-Flk\text{-}1^+$ cell population in the peripheral blood were isolated by FACS and were cultured in vitro with 100 nM VEGF supplemented medium for 10 d. Then, 2 μg of DiI-labeled acetylated LDL (2 μg/ml) was added to cultured cells, and incorporation of DiI-labeled acetylated LDL was observed with a co-staining by anti-eNOS antibody followed by FITC-labeled anti-rabbit IgG antibody. Ten percent of plated cells adhered to plastic dishes and expanded. Also, 50% of growing cells exhibited the ability to incorporate DiI-acetylated LDL and simultaneously expressed eNOS (white arrow in the merged picture). (B) Differentiation of $CD3^-B220^-Flk\text{-}1^+$ cells into endothelial cells in vivo. The $CD3^-B220^-Flk\text{-}1^+$ cells over-expressing EGFP were isolated with FACS from the peripheral blood of EGFP transgenic mice that had already been undergone the limb ischemic operation 3 d before. 2×10^6 $CD3^-B220^-Flk\text{-}1^+$ cells were then intravenously transplanted into mice that had already undergone hindlimb ischemic operation at 1 d before transplantation. Seven days after transplantation, ischemic and normal hindlimb muscle was removed and stained with an endothelial cell marker, an anti-CD31 antibody. EGFP-positive cells were detected between the muscle fiber only in the ischemic hindlimb muscle and were simultaneously positive for CD31 (white arrowheads in the merged picture). Each experiment was repeated for four times with similar results and representative data were shown.

[33,34]. Cao et al. [35] showed the evidence that IL-1β lowered prolyl-hydroxylase activity in cultured cell, leading to increased amounts of HIF-1α. Thus, it is conceivable that the lack of IL-1β leads to an increase in the activity of prolyl-hydroxylase, which causes more degradation of HIF-1α and impairs an increase in its amount. Furthermore, the Src–PI3K–Akt-1 pathway has recently been shown to upregulate HIF-1α function by increasing HIF-1α transcription. Akt-1 can be activated by the IL-1β receptor signaling pathway associated with the activation of tyrosine kinase FAK and the subsequent activation of PI3K under ischemic conditions [36–39]. We observed that the activation of FAK and Akt-1 in hindlimb muscle at 1 d after ischemia in IL-1β–/– mice was weaker than in the wild type (Amano et al., unpublished observation). Thus, the decreased activation of FAK and Akt-1 may partly contribute to the decreased amount of HIF-1α in ischemic tissue.

In summary, the present study demonstrates the critical role of inflammatory cytokine IL-1β to enhance neocapillary formation in limb ischemia. IL-1β-mediated accumulation of HIF-1α, expression of VEGF, its receptor and adhesion molecules and mobilization of $CD34^-Flk\text{-}1^+$ EPCs are closely involved in IL-1β-induced neovascularization.

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Conflict of Interest: The authors have declared that no conflict of interest exists.

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