The Rho kinase inhibitor fasudil augments the number of functional endothelial progenitor cells in *ex vivo* cultures

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Abstract. Rho kinase (ROCK) has been implicated in the regulation of vascular tone, endothelial dysfunction, inflammation and remodeling. Endothelial progenitor cells (EPC) have been proven to have the efficacy of therapeutic neovascularization in ischemia. However, the scarcity of EPCs limits cell therapy. Using an *in vitro* EPC culture assay, Y27632 was found to increase the number of adherent EPCs. In this study, we investigated the effect of fasudil, another ROCK inhibitor being used in the clinic, on EPC number and examined whether EPCs expanded by fasudil are functional *in vitro* and *in vivo*. In *ex vivo* cultures of EPCs, fasudil effectively increased the number of ac-LDL/UEA-1 positive cells as well as adherent cells, in contrast to H89, a less selective ROCK inhibitor. Fasudil also increased EPC numbers in culture up to 10 μM, in a dose-dependent manner. When EPCs expanded with fasudil were examined for the migratory activity toward stromal cell-derived factor-1 and vascular endothelial growth factor, these cells retained functional properties in migration, albeit with some decrease. Fasudil-cultured EPCs labeled with PKH26 showed an activity similar to non-treated EPCs for cellular adhesion into an endothelial cell (EC) monolayer and incorporation into capillary-like structures formed by ECs. Finally, when EPCs cultured with fasudil (10^6 cells/mouse) were injected into ischemic limbs, these cells showed a blood flow recovery at a level comparable to non-treated control EPCs and increased neovascularization. Therefore, these data suggest that the ROCK inhibitor fasudil can provide a beneficial effect in the treatment of ischemic diseases by increasing EPC numbers.

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Abbreviations: BS, *Griffonia (Bandeiraea) simplicifolia*; BSA, bovine serum albumin; EPC, endothelial progenitor cell; DiI-acLDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein; HUVEC, human umbilical vein endothelial cell; KDR, kinase insert domain receptor; LDPI, laser Doppler perfusion imaging; MNC, mononuclear cell; ROCK, Rho kinase; SDF-1, stromal cell-derived factor-1; siRNA, small interfering RNA; UEA-1, *Ulex europaeus* agglutinin-1; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor

Key words: Rho kinase inhibitor, endothelial progenitor cell, fasudil, ischemia, neovascularization

Introduction

Substantial evidence suggests that endothelial progenitor cells (EPCs) exist within the total population of mononuclear cells and play a crucial role in neovascularization of ischemic tissue (1,2). Ischemia can trigger the release of bone marrow derived EPCs into the periphery and the mobilized EPCs then contribute to new blood vessel formation (3). Cytokines such as the granulocyte macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), and stromal cell-derived factor-1 (SDF-1) were reported as potent stimulators of EPC mobilization (3-5). However, the scarcity of EPCs in the bone marrow and in peripheral blood could limit the cell therapy of EPCs. EPC amplification is an important issue in cell therapy. In an attempt to find pharmacological substances that increase EPC number, among various tested inhibitors we identified the Rho kinase (ROCK) inhibitor, Y27632 that exhibited such activity.

The small GTPase Rho, and its downstream effector, ROCK have been implicated in many of the pathogenic processes such as endothelial dysfunction, vasoconstriction, inflammation, cellular migration and proliferation and a procoagulant state (6). At the cellular level, the Rho/ROCK pathway is important in controlling migration, proliferation, differentiation, apoptosis, survival and gene transcription. In endothelial cells, inhibition of Rho/ROCK has been shown to augment the expression and activity of endothelial nitric oxide synthase (eNOS) (7-9), and to improve endothelial function in coronary artery disease patients (10). The inhibition of ROCK has been shown to have a beneficial effect in a variety of cardiovascular disorders including angina, ischemic stroke, ischemia-reperfusion injury and heart failure (11-13).

Lipid lowering agents such as HMG CoA reductase inhibitors or statins elicit the cholesterol-independent or 'pleiotrophic' effects in part mediated by inhibition of ROCK (14,15). Statins have also been reported to increase of EPC proliferation and mobilization, although not in a ROCK/endothelial nitric oxide (NO) synthesis pathway (16,17). Interestingly, Y27632, a ROCK inhibitor increased EPC number in our *in vitro* culture condition.

Among the ROCK inhibitors, fasudil has been proven in clinical studies to offer benefits in ischemic stroke and vasoconstriction by inhibiting ROCK (12). Since Y27632 showed a marked effect on the increase of EPC number in our studies, we compared several ROCK inhibitors including fasudil in terms of their ability to enhance the EPC number in *ex vivo* culture, and investigated whether this increase in the number of EPCs by fasudil is functional and useful for cell therapy.

Materials and methods

Cell culture. Human umbilical vein endothelial cells (HUVECs) were isolated from human cords as previously described (18), maintained in 20% FBS (Gibco-BRL, Grand Island, NY), 30 μg/ml endothelial cell growth supplements (Sigma, St. Louis, MO, USA), 90 μg/ml heparin (Sigma) and 1% antibiotics in M199 (Gibco-BRL), and used at passages 3-8.

For EPC culture, human umbilical cord blood samples were collected in sterile blood bag (Green Cross, Korea) containing heparin as the anticoagulant. Written informed consent was obtained from all mothers and all procedures were approved by the Institutional Review Board at The Catholic University of Korea, College of Medicine (approval no. CUMC10U901). Our study conformed to the principles outlined in the Declaration of Helsinki for use of human tissue or subjects. Mononuclear cells (MNCs) were isolated from the cord blood sample by a Ficoll-Paque Premium (density of 1.077 g/ml; GE Healthcare, NY, USA) density gradient centrifugation method as previously described (19). Isolated MNCs (2,500/mm^2) were seeded on culture dishes coated with 20 μg/ml fibronectin (FN) and maintained in the HUVEC medium. After 3 days, non-adherent cells were removed by washing with PBS and adherent cells were further cultivated until day 5-7.

Characterization of differentiated adherent EPCs. To characterize the cells as having features of cells of the endothelial lineage, we examined the adherent cells for their ability to uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiI-acLDL) (Invitrogen, Eugene, Oregon) and to bind to *Ulex europaeus* agglutinin-1 (UEA-1, Sigma) as previously described (19).

Fluorescence activated cell sorting (FACS) analysis was performed for the assessment of other endothelial cell marker expression of EPCs cultured in the presence or absence of fasudil for 6 days. The cultured cells were isolated from culture plates by incubating with 1 mM EDTA (pH 7.4) in PBS for 3-5 min at 37˚C, and Fc receptors and nonspecific binding of immunoglobulin were blocked by human IgG (Sigma) treatment. The cells (2x10^5) were then incubated at 4˚C for 30 min in the dark with phycoerythrin (PE)-conjugated monoclonal antibodies against CD34 (BD Pharmingen, San Jose, CA, USA) or against vascular endothelial-cadherin (VE-Cadherin, R&D Systems), or a FITC-conjugated monoclonal antibody against kinase insert domain receptor (KDR, R&D Systems, Minneapolis, MN, USA), or incubated with monoclonal antibodies against CD31 (Dako, Glostrup, Denmark) and the von Willebrand factor (vWF, Dako) followed by incubation with a secondary antibody conjugated with Cy3 (Chemicon, Temecula, CA). Cells to be stained with vWF were permeabilized with 90% cold methanol at -20˚C for 20 min before incubation with the primary antibody. The immunofluorescence-labeled cells were washed with FACS buffer (0.5% BSA and 0.09% sodium azide in PBS, pH 7.4) and analyzed by quantitative flow cytometry using FACSCalibur (BD Biosciences, San Jose, CA, USA). The CellQuest software was used for counting 10,000 events/sample. Isotype-identical directly or indirectly conjugated antibodies served as negative controls (IgG_1-FITC for KDR, IgG_1-PE for CD34, IgG_{2B}-PE for VE-Cadherin, and IgG_1 for CD31 and vWF).

Modified Boyden chamber migration assay. Chemotactic migration was evaluated using a modified Boyden chamber as previously described (20). After incubation in serum-free medium for 4 h, day 6 EPCs cultured with fasudil (1~10 μM) or simvastatin (0.01 μM) were detached and placed into the upper chamber (2x10^4 cells/well). The low chamber was filled with buffer containing 0.1% BSA, 90 μg/ml heparin, and SDF-1 or VEGF (10 ng/ml) in M199 medium. The assembled chamber was incubated for 6 h at 37˚C with 5% CO_2 to allow cells to migrate through the filter. The membrane was removed from the chamber, and stained with Diff-Quik solution (Sysmex, Japan). Non-migrated cells of the upper surface of the membrane were removed. The number of migrated cells was counted in random four fields (x200) of each well. Each experiment was performed in triplicate.

Cell adhesion assay. HUVECs were seeded in 48-well plates by adding 5x10^4 cells/well 24 h before the assay. Confluent HUVEC monolayers were stimulated for 12 h with endothelial growth medium-2 SingleQuots (EGM-2 SingleQuots) (Cambrex, Walkersville, MD). EPCs cultured in the presence or absence of fasudil (10 μM) for 6 days were labeled with PKH26 (Sigma) according to the manufacturer's instruction. PKH26-labeled EPCs (1x10^5/well) were then added to the HUVEC monolayer. After 3 h of incubation at 37˚C, the cells were washed twice with PBS to remove non-adherent cells, fixed with 4% paraformaldehyde, and stained with DAPI. The PKH26-labeled EPCs adhering to HUVEC layer were quantified in triplicates.

Tube formation assay. Day 7 EPCs were detached with 1 mM EDTA and labeled with PKH26 (Sigma) according to the manufacturer's instructions. PKH26-labeled EPCs (7,500 cells) were mixed with HUVECs (3x10^4 cells), then seeded onto Matrigel-coated 48-well plates and incubated at 37˚C with 5% CO_2 for 22 h. Tube formation was visualized using a phase-contrast microscope, and then the image was overlaid with fluorescence microscopic images (Carl Zeiss).

Transplantation in a hindlimb ischemia murine model. All procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised 1996). The study

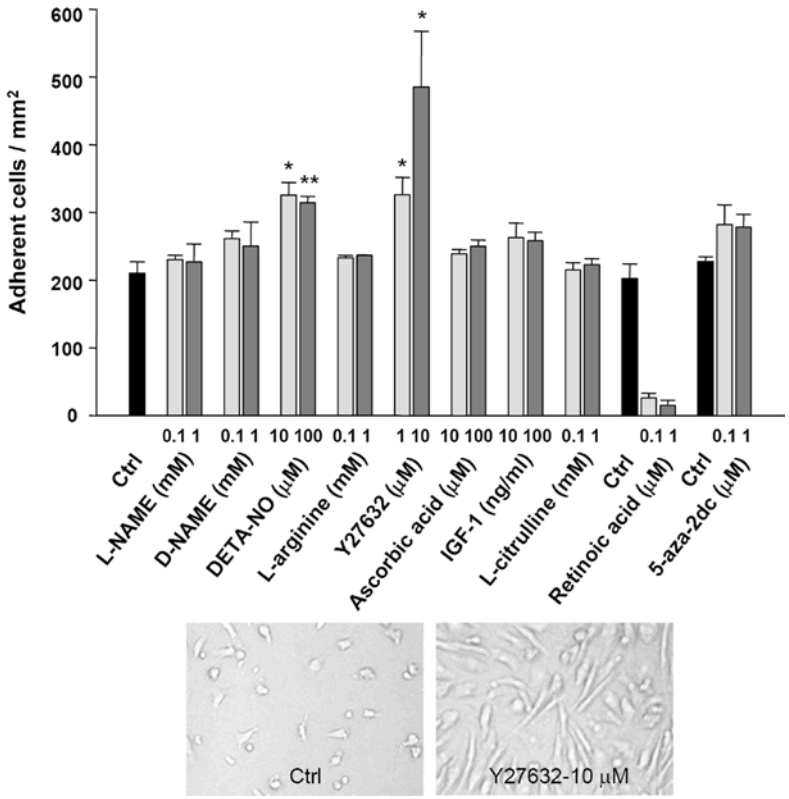

Figure 1. Y27632 markedly increases the number of adherent EPCs. MNCs were seeded on the FN-coated plates at a density of 2,500 cells/mm^2 and incubated in the presence of each chemical for 7 days. The number of adherent cells was counted. Each value represents the mean ± SE. *P<0.05; **P<0.01 compared with each relevant solvent control (Ctrl).

protocol was approved by our Institutional Animal Care and Use Committee.

Male Balb/c Slc-nu mice (Japan SLC, Inc., Japan), 8-weeks of age were anesthetized with an i.p. injection of 100 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. Hindlimb ischemia was induced by operative resection of one femoral artery and boundary vessels. No later than 6 h after operation, one group of mice was transplanted with day 5-6 EPCs (10^6 cells/mouse) cultured in the presence or absence of fasudil (10 μM) administered intramuscularly in the ischemic thigh area. Three weeks after surgery, the mice were sacrificed by CO_2 inhalation. The sections (5 μm) of the tissues that were harvested from ischemic limbs, were stained with fluorescein *Griffonia (Bandeiraea) simplicifolia* (BS) lectin I (Vector Laboratories, Burlingame, CA), as follows: 5 μm muscle tissues were fixed with 100% acetone for 5 min, washed with PBS twice, blocked with 5% normal goat serum (Vector Laboratories) for 30 min, and incubated with 20 μg/ml fluorescein BS lectin I for 1 h at room temperature. To quantify the vessel density, ImageJ software was used (http://rsbweb.nih.gov/ij/). First, the captured color images were applied to the RGB stack to split the images into each channel. After segmenting each individual image using thresholding (CTRL-EPC group, mice (n=5), 10 sections/mouse, 5 fields/section, total 50 images calculated; fasudil-EPC group, mice (n=5), 10 sections/mouse, 5 fields/section, total 49 images calculated), the percentages of the vessel area/fields were calculated by ImageJ.

Laser Doppler perfusion imaging of the hindlimb blood flow. Laser Doppler perfusion imaging (LDPI; Perimed PeriScan PIM3, Stockholm, Sweden) was used to measure the ratio of the ischemic (left)/non-ischemic (right) limb blood flow over the course of 3 weeks postoperatively. After scanning over the same region of interest in each mouse twice, images were subjected to quantification of the blood flow and the averages of the blood flow of the ischemic and non-ischemic limbs were calculated.

Statistical analysis. All values are presented as the means ± SE from at least three independent experiments. Statistical analysis was performed using the Student's t-test. Probability values <0.05 were considered to be statistically significant.

Results

ROCK inhibitors increase the number of EPCs. In order to find a way of increasing EPC number and improving their functions by avoiding cellular senescence, we screened several chemicals in *in vitro* EPC culture by β-gal senescence assay. Most of the chemicals tested apparently affected EPC senescence during 7 day culture (data not shown). Interestingly, Y27632, a ROCK inhibitor, gave rise to marked increased adherent cells when MNCs isolated from cord blood were cultured on FN-coated dishes for 7 days in the presence of each chemical (Fig. 1). NO donor also slightly increased EPC number. NO has been shown to increase neovascularization *in vivo*, and various agents that promote eNOS-dependent NO production have been shown to induce therapeutic angiogenesis (21). However, retinoic acid strongly inhibited adhesive differentiation of EPCs.

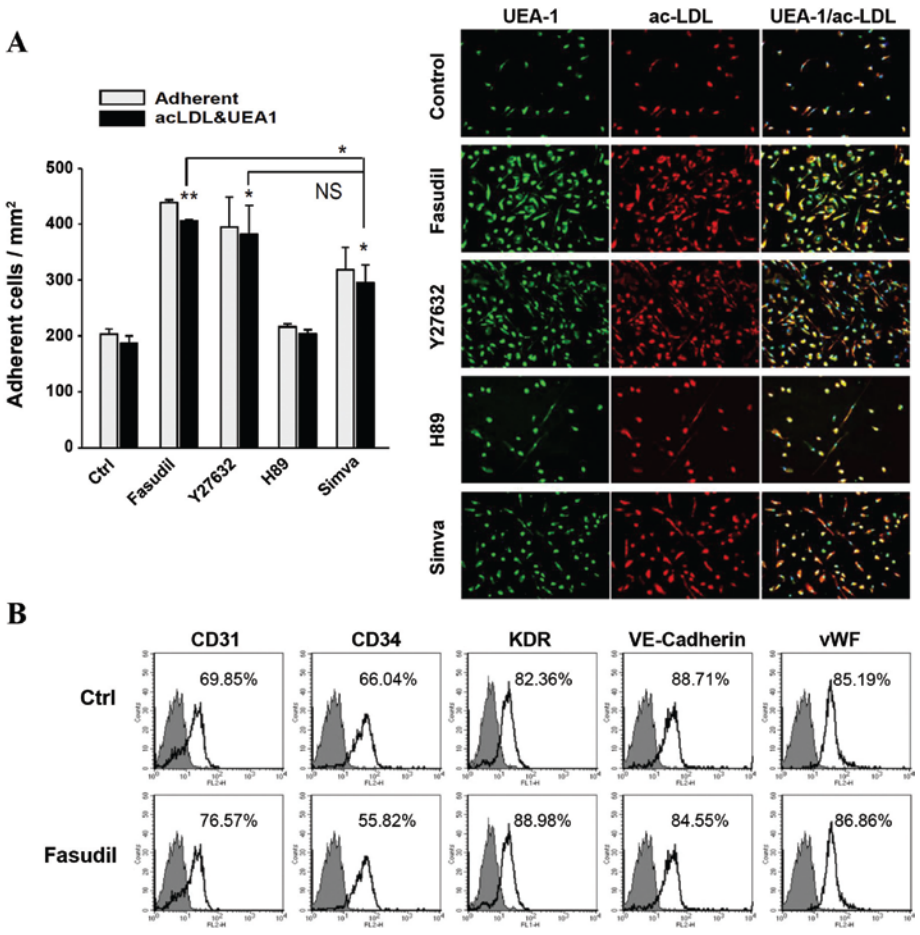

Figure 2. Rho kinase inhibitors with a higher selectivity increase the number of EPCs with greater efficiency. (A) MNCs were incubated in the presence of fasudil, Y27632, H89 (10 μM) or simvastatin (Simva, 1 μM) for 7 days and then adherent EPCs were assessed for the uptake of acLDL (red) and binding of UEA-1 (green). The number of adherent EPCs and acLDL/UEA-1 double positive cells was counted. Each value represents the mean ± SE. NS, not significant; *P<0.05; **P<0.01 compared with acLDL/UEA1 positive control cells. Representative photographs are shown. (B) Day 6 EPCs cultured in the presence or absence of fasudil (10 μM) were immunolabeled and analyzed for expression of endothelial markers by FACS analysis.

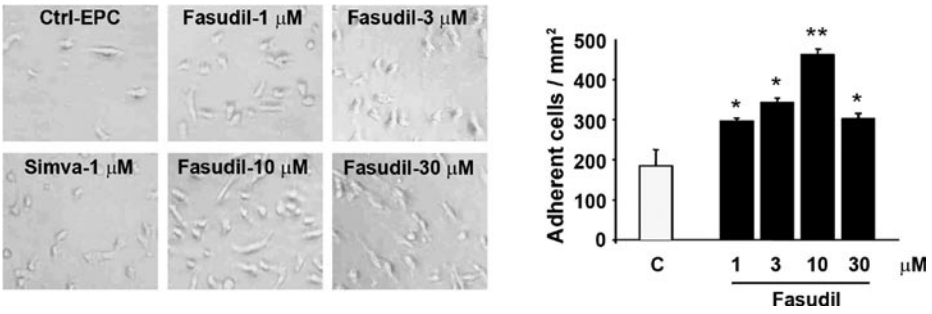

Figure 3. Fasudil dose-dependently increases EPC number. MNCs were incubated in the presence of fasudil in a concentration range of 1-30 μM for 7 days and the number of adherent EPCs was measured. Each value represents the mean ± SE. NS, not significant; *P<0.05; **P<0.01 compared with PBS-treated control cells.

Next, we compared several inhibitors with different levels of selectivity for ROCK and other kinases in EPC culture. Y27632 and fasudil that are the most selective ROCK inhibitors, increased the number of EPCs by ~2-fold compared to PBS-treated controls, whereas a less selective ROCK inhibitor H89, which strongly inhibits protein kinase A, S6 kinase 1 and the mitogen- and stress-activated protein kinase 1 as well as ROCK, had no effect on increasing the number of EPCs (Fig. 2A). A higher number of spindle-shaped cells was observed in fasudil-cultured cells compared to statin- or PBS-treated cells. Most of the adherent cells were double positive for ac-LDL uptake and UEA-1 binding, without a change in the ratio upon treatment with the inhibitor. The enhancing effect of fasudil was distinctly higher than that of simvastatin (P<0.05). When the expression levels of other endothelial cell markers, CD31, CD34, KDR, VE-Cadherin and vWF, were compared by FACS analysis of immunolabeled cells after culture for 6 days, there were no noticeable differences

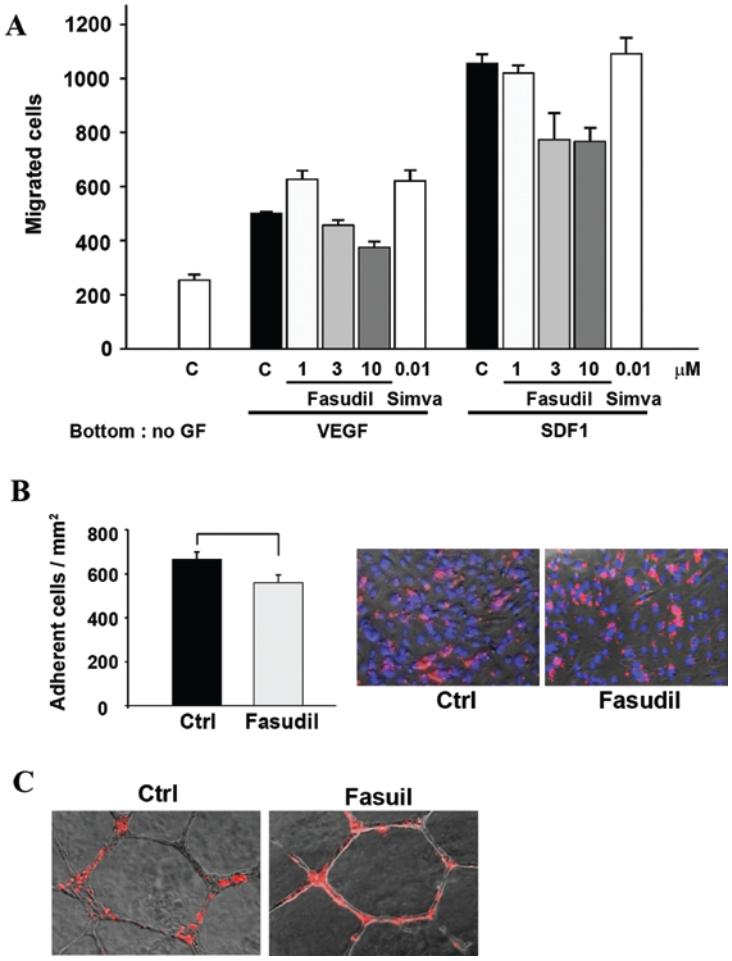

Figure 4. Fasudil-expanded EPCs retain most of the functional properties of non-treated EPCs. (A) MNCs were cultured in the presence or absence of fasudil (1~10 μM) or simvastatin (Simva; 0.01 μM) for 6 days. These cells were induced to migrate toward VEGF or SDF-1 (10 ng/ml) in a modified Boyden chamber. The number of migrated cells was determined. (B) Day 6 EPCs cultured in the presence of fasudil (10 μM) were labeled with PKH26 (red) and then the labeled EPCs (10^5 cells/48-well plates) were added onto a confluent HUVEC monolayer, followed by incubation for 3 h. After washing with PBS, the cells were photographed in five fields/each well and the number of the labeled cells adhering to HUVEC layer was counted. Each value represents the mean ± SE. *P<0.05 vs. control. Representative fields are shown (x200). (C) Day 6 EPCs (7,500 cells) cultured in the presence of fasudil (10 μM) were labeled with PKH26 (red), mixed with HUVECs ($3x10^4$ cells), and then incubated on Matrigel for 22 h. Representative fields are shown (x100).

between cells cultured in the presence or absence of fasudil (Fig. 2B).

In order to examine whether the effect of fasudil was dose-dependent, EPCs were treated for 7 days with 1-30 μM fasudil (Fig. 3). Treatment of fasudil increased the number of adherent EPCs dose-dependently up to 10 μM. However, treatment with the high concentration of 30 μM resulted in reduction of the adherent EPCs. From these results, we conclude that ROCK inhibition by fasudil increases the number of EPCs in an *ex vivo* culture.

Fasudil-expanded EPCs retain the functional properties of EPCs. ROCK inhibitors can either inhibit or enhance cell migration depending on the cell type and conditions (22). Thus, we examined whether EPCs increased upon treatment with fasudil would retain the functional properties of EPCs. When EPCs cultured in the presence of fasudil for 6 days were assayed in a modified Boyden chamber, these cells were able to migrate in response to SDF-1 or VEGF albeit at lower levels compared to non-fasudil-treated cells (Fig. 4A). Even after culture with 10 μM of fasudil, EPC migration was much higher than at the non-stimulated basal level (P<0.05). Next, we assessed whether fasudil-cultured EPCs have a capacity to bind to activated endothelial cells. In an adhesion assay, fasudil-cultured EPCs were able to adhere to the HUVEC monolayer at 83.87±5.36% of control EPC level (Fig. 4B). Finally, we examined whether fasudil-cultured EPCs retain the ability to incorporate into endothelial tubes. When EPCs cultured in the presence of fasudil was co-incubated with HUVECs on Matrigel, fasudil-cultured EPCs labeled with PKH26 were able to incorporate into tubes formed by HUVECs at a similar level to non-treated control EPCs (Fig. 4C). Thus, these results indicate that fasudil-cultured EPCs retain most of their functional properties. Thus, it could be expected that fasudil-cultured EPCs may function normally without any significant inhibition *in vivo*.

Fasudil-cultured EPCs show in vivo efficacy at a level comparable with control EPCs in hindlimb ischemia. Finally, we compared the therapeutic effects of fasudil-cultured EPCs with non-treated EPCs in an *in vivo* ischemic model, to evaluate the possibility of application of fasudil in cell therapy. Within

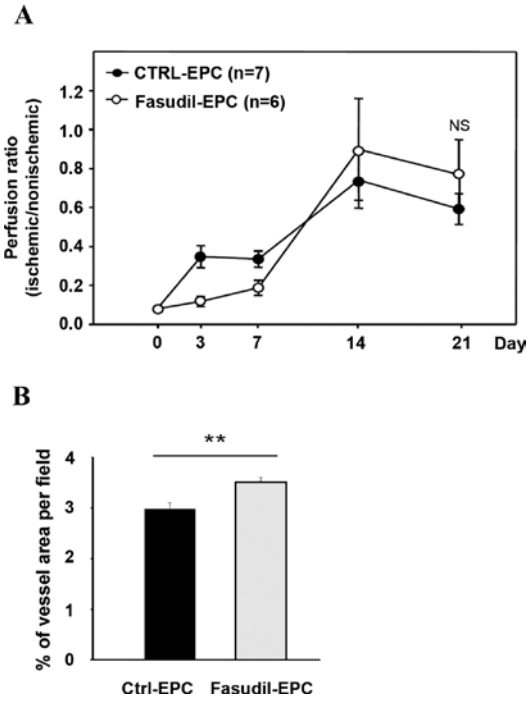

Figure 5. Comparison of blood flow recovery in hindlimb ischemia between fasudil-expanded EPCs and control EPCs. (A) EPCs were cultured with or without fasudil for 5-6 days. EPCs ($1x10^6$) were intramuscularly injected into the ischemic muscle area. Perfusion was quantified by LDPI as described in Materials and methods. (B) The sections of tissues harvested from ischemic limbs after 3 weeks were stained with fluorescein BS lectin I and the vessel density was measured as described in Materials and methods. NS, not significant; $^{**}P<0.01$.

6 h after operation, EPCs cultured with or without fasudil for 5-6 days were locally injected into the ischemic thigh muscle area (10^6 cells) at four different injection points. Serial LDPI analyses showed overall similar levels of recovery of blood flow between control EPCs and fasudil-cultured EPCs, but slightly better recovery was observed in fasudil-cultured EPCs on days 14 and 21 by showing higher mean augmented ratios of the ischemic/non-ischemic limb blood flow in the limbs injected with fasudil-cultured EPCs (Fig. 5A). When tissue sections harvested from the ischemic hindlimb at day 21 after operation were inspected by BS lectin I staining, an increase in vessel density was observed in the fasudil-cultured EPC group compared to the control-EPC group (Fig. 5B). Therefore, the results suggest that fasudil-cultured EPCs are effective in therapeutic vascularization *in vivo*.

Discussion

The data from the present study establish a novel effect of ROCK inhibitors in EPCs in *ex vivo* culture. Fasudil increased EPC number and fasudil-expanded EPCs retained most of the functional characteristics of control EPCs. Therefore, this study suggests that fasudil may be used for *ex vivo* expansion of EPCs for cell therapy.

Y27632 and fasudil are relatively more selective for ROCK compared to H89 (23). Accordingly, they showed a better effect on EPCs by enhancing the yields of adherent EPCs in an *ex vivo* culture assay. Simvastatin also increased EPC number, in accordance with a previous report (17), but the increased level by simvastatin was lower than that by fasudil treatment. Since gene knockdown experiments in EPCs with small interfering RNA (siRNAs) failed, we cannot conclude that ROCK inhibition results in an increase of EPC number at the molecular level. However, ROCK inhibitors may be beneficial in EPC culture, since only the more selective ROCK inhibitors effectively enhanced the number of EPCs. Another group has reported that fasudil modifies the detrimental effect of TNF-α on human peripheral blood EPCs by recovering EPC number (24), supporting our notion that fasudil positively affects EPC number. However, in a previous study, treatment of fasudil for 24 h did not noticeably augment EPC number when MNCs from the peripheral blood of healthy human volunteers were treated (16). Therefore, the treatment period of the inhibitor and the prior cell environment, such as the exposed cytokine composition may influence the effect of a ROCK inhibitor on EPC number.

ROCK is involved in the actin-cytoskeleton rearrangement and therefore affects cell motility (22). ROCK inhibitors can either inhibit or enhance cell migration depending on the cell type and the conditions. Fasudil-expanded EPCs show a comparable migratory activity and adhesion with non-treated EPCs although with some level of inhibition. In addition, incorporation into HUVEC-derived tubes was not affected by fasudil treatment in EPC culture. Fasudil-expanded EPCs were also comparably effective in therapeutic vascularization and perfusion recovery after ischemia. Thus, the data strongly support that fasudil can be used for EPC expansion.

The clinical beneficial effect of fasudil on ischemic diseases, such as stroke has been described. However, the mechanisms underlying the fasudil effect are not fully understood. Amelioration of endothelial damage/dysfunction has been suggested as a possible mechanism for the neuroprotective effects of ROCK inhibitors against ischemic brain damage (25). Leukocyte ROCK activity is increased after ischemic stroke (26), and anti-inflammation through inhibition of leukocytes by fasudil may be another possible mechanism. However, a population of leukocytes may be the EPCs with the increased ROCK activity. Notably, inhibition of RhoA GTPase activity has been reported to enhance hematopoietic stem and progenitor cell proliferation and engraftment, although with reduced migration and adhesion (27). Thus, it can be suggested that EPCs may be a target of ROCK inhibitors in ischemia. Taken together, these results provide insight for the usefulness of fasudil in ischemic disease.

Acknowledgements

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