Erythropoietin-Mobilized Endothelial Progenitors Enhance Reendothelialization via Akt–Endothelial Nitric Oxide Synthase Activation and Prevent Neointimal Hyperplasia

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Abstract—We investigated whether the mobilization of endothelial progenitor cells (EPCs) by exogenous erythropoietin (Epo) promotes the repair of injured endothelium. Recombinant human Epo was injected (1000 IU/kg for the initial 3 days) after wire injury of the femoral artery of mice. Neointimal formation was inhibited by Epo to 48% of the control (P < 0.05) in an NO-dependent manner. Epo induced a 1.4-fold increase in reendothelialized area of day 14 denuded vessels, 55% of which was derived from bone marrow (BM) cells. Epo increased the circulating Sca-1/Flk-1/CD34 EPCs (2.0-fold, P < 0.05) with endothelial properties NO dependently. BM replacement by GFP- or lacZ-overexpressing cells showed that Epo stimulated both differentiation of BM-derived EPCs and proliferation of resident ECs. BM-derived ECs increased 2.2- to 2.7-fold (P < 0.05) in the Epo-induced neendothelium, where the expression of Epo receptor was upregulated. Epo induced Akt/eNOS phosphorylation and NO synthesis on EPCs and exerted an antiapoptotic action on wire-injured arteries. In conclusion, Epo treatment inhibits the neointimal hyperplasia after arterial injury in an NO-dependent manner by acting on the injured vessels and mobilizing EPCs to the neo-endothelium. (Circ Res. 2006;98:1405-1413.)

Key Words: restenosis ■ endothelium ■ progenitor cells ■ erythropoietin

Endothelial cells (ECs) cover the luminal surface of blood vessels and maintain multiple vascular functions. Disruption of endothelial coverage causes a decrease in the production of vasculoprotective mediators such as nitric oxide (NO), leading to elevated vascular tone, enhanced inflammation and medial smooth muscle cell proliferation. The resultant neointimal hyperplasia causes restenosis in various pathological conditions.1

Bone marrow (BM)-derived endothelial progenitor cells (EPCs) have been isolated from the mononuclear cell (MNC) population in peripheral blood (PB).2,3 They have differentiated into ECs,2 suggesting that they may have a potential to accelerate reendothelialization. Recently, transplantation of autologous PB EPCs to balloon-denuded arteries was reported to facilitate reendothelialization of the injured artery.4,5 Intravenous transfusion of spleen-derived EPCs or EPCs overexpressing eNOS reduces neointimal formation after vascular injury.6,7 Delivery of primary cultured PB MNCs to balloon-injured arteries leads to accelerated reendothelialization to promote endothelium-dependent vasoreactivity.8

Erythropoietin (Epo) stimulates the proliferation and differentiation of erythroid lineage progenitors. Mature ECs express Epo receptors (EpoRs),9 and Epo induces proangiogenic response in cultivated mature ECs, as evidenced by EC proliferation and migration10 and the antiapoptotic effect on ECs11 as well as NO production.12 Epo increases circulating EPCs to stimulate neovascularization in vivo13 or induces proangiogenic phenotype in cultured ECs14 and also improves wound healing by angiogenesis in the genetically diabetic mouse.15 This evidence leads to the hypothesis that Epo may provide an effective noninvasive strategy to enhance reendothelialization of injured vessels.

Several studies have shown that the exogenous administration of cytokines increases the number of circulating EPCs. For example, pretreatment with vascular endothelial growth factor (VEGF) was reported to double the number of circulating EPCs in humans,16,17 and the administration of granulocyte colony–stimulating factor (G-CSF) recruited EPCs from BM.17 Mobilization of the circulating EPCs by exogenous G-CSF facilitates endothelialization and inhibits neointimal development.18

In this study, we evaluated the efficacy of short-term Epo treatment as a strategy for promoting reendothelialization followed by the inhibition of neointimal hyperplasia in
wire-injured arteries. Our results show that only 3-day treatment with Epo causes mobilization of circulating CD45dim/Flk-1+ or Sca1+/Flk-1+ EPCs and stimulates both differentiation of BM-derived EPCs on the endothelial layer and proliferation of resident ECs associated with endothelial EpoR-mediated activation of the Akt-eNOS pathway and SMC antiapoptotic effect, resulting in a marked inhibition of neointimal formation.

**Materials and Methods**

**Vascular Injury, Epo Administration, and Morphometric Analysis**

Transluminal arterial injury was performed in 8-week-old male C57BL/6 mice. A straight spring wire (0.25 mm in diameter) was inserted into the left femoral artery and placed there for 3 minutes. This wire injury was reported to cause a complete removal of endothelium. Human Epo (Chugai, Tokyo) (1000 IU/kg body weight) or saline was injected intraperitoneally just after arterial injury and once daily for the following 2 days. Treatment with Nω-nitro-l-arginine methyl ester (l-NNAME) (3.7 mmol/L) or 2.25% l-arginine hydrochloride (106.8 mmol/L) (Sigma) in the drinking water took place for 7 days before wire injury and continued for 14 days after wire injury.

The dose of Epo was determined based on the previous report. The injured arteries were harvested at day 14 and fixed with 4% paraformaldehyde. Paraffin-embedded sections were stained with Elastica van Gieson. Three sections from each artery at 300-μm intervals were analyzed using ImageJ 1.32q software (NIH). In other animals, Evans blue dye (5%; Sigma) was transfused to mice 10 minutes before euthanasia to identify the remaining denuded area 5 and 14 days after wire injury. After removal, arterial tissues were longitudinally opened and then placed on slide glasses to take pictures under microscope (MS5 Olympus). All animal procedures were approved by institutional guidelines. The collection of blood samples and the consent protocol for the volunteers was approved by institutional guidelines.

**Fluorescence-Activated Cell Sorting**

PB (100 μL) was collected 3 days after injury and incubated for 15 minutes with anti-mouse CD34-fluorescein isothiocyanate (FITC), Flk-1-PE, Sca1-FITC, CD45-PECy5 antibodies (BD Pharmingen). After erythrocyte lysis, cells were analyzed with FACS Caliber (Becton Dickinson). CD45dim/Flk-1+ cells were sorted with FACSDiva software (Becton Dickinson) with purity of sorted cells assessed by fluorescence-activated cell sorter (FACS) analysis grater than 90%. The CD133+ progenitor cells were cultured in EBM-2 medium supplemented with 5% FBS, EGM-2-MV-SingleQuots (Clonetics) a day before analysis, as described below. All cells were maintained at 37°C in a humidified incubator at 5% CO2. After serum starved in DMEM supplemented with 0.5% FBS for 12 hours, they were stimulated with 1.2 IU/mL of Epo for 30 minutes and fixed. Immunofluorescence was performed by using antibodies against EpoR (sc-697; Santa Cruz Biotechnology) with phosphorylated Akt and eNOS (Cell Signaling Technology). For double staining, we used Zenon rabbit IgG labeling kits (Molecular Probes) only when needed.

**Murine Cell Culture Assay**

The sorted CD45dim/Flk-1+ cells were cultured with EBM-2 medium supplemented with 5% FBS, EGM-2-MV-SingleQuots (Clonetics), and 10 ng/mL VEGF (Peprotech) on fibronectin-coated chamber slides (Becton Dickinson). Adherent cells were reseeded after 4 days and maintained for 7 days. The culture cells were incubated with 2.4 μg/mL Alexa Fluor 594-labeled acetylated LDL (aLDL) (Molecular Probes) for 120 minutes, fixed with 2% paraformaldehyde, and incubated with 10 ng/mL of BS-1-FITC (Sigma) for 1 hour, and double-fluorescent cells were counted as EPCs in 4 randomly selected fields under confocal microscopy (FLUOVIEW BX50; Olympus).

**Immunohistochemistry**

Arteries were harvested on day 14 and fixed in 4% paraformaldehyde. Paraffin cross-sections were stained with antibodies against CD31 (sc-8306; Santa Cruz Biotechnology) and EpoR (sc-5624 or sc-697) followed by the avidin–biotin complex technique and diaminobenzidine substrate (Vector Laboratories). Sections were counterstained with hematoxylin. Frozen sections were stained with antibodies (GFP-Alexa Fluor 488; Molecular Probes; CD31-PE, BD Pharmingen), followed by Alexa Fluor 633–conjugated anti-rabbit IgG secondary antibody (Molecular Probes), and then observed under the confocal microscopy. The number of fluorescent cells from 6 sections was evaluated statistically.

To detect apoptotic cells in situ, TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining was performed for paraffin sections using the In-Situ Cell Death Detection Kit (Chemicon International Inc) and counterstained with methyl green.

**Human Cell Isolation and Culture**

PB was obtained. PB MNCs from venous blood of healthy human volunteers were isolated by density-gradient centrifugation (Lymphoprep; Axis Shield). CD133+ progenitor cells were purified from PB MNCs by positive selection with anti-CD133+ microbeads respectively using magnetic cell sorter device (Miltenyi Biotec). The purity of sorted cells assessed by fluorescence-activated cell sorter (FACS) analysis was grater than 90%. The CD133+ progenitor cells were cultured in EBM-2 medium supplemented with 5% FBS, EGM-2-MV-SingleQuots (Clonetics) a day before analysis, as described below. All cells were maintained at 37°C in a humidified incubator at 5% CO2. After serum starved in DMEM supplemented with 0.5% FBS for 12 hours, they were stimulated with 1.2 IU/mL of Epo for 30 minutes and fixed. Immunofluorescence was performed by using antibodies against EpoR (sc-6924; Santa Cruz Biotechnology) with phosphorylated Akt and eNOS (Cell Signaling Technology). For double staining, we used Zenon rabbit IgG labeling kits (Molecular Probes) only when needed.

For measurement of the intracellular NO level, the cells were loaded with 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (10μmol/L; Daiichi Pure Chemicals, Tokyo, Japan) for 30 minutes at 37°C in the dark, washed twice with buffer, incubated for another 30 minutes, and then visualized under laser microscopy.

Fluorescent intensity was evaluated with Adobe Photoshop software.

**Measurement of NOx Concentration**

NOx concentration in the serum was measured with a high-performance liquid chromatography (HPLC) Griess system, as previously described.

**Statistics**

Statistical analyses were performed with 1-way ANOVA followed by pair-wise contrasts using the Dunnett’s test. Data are expressed as means±SE for continuous variables. P<0.05 was considered statistically significant.

**Results**

**Epo Inhibits Neointimal Hyperplasia**

A prominent, concentric neointima developed in the saline-treated vessels 14 days after injury, whereas neointimal formation was markedly reduced in the Epo-treated animals (Figure 1A). Morphometric analysis of serial sections (Figure 1B) showed a marked decrease (52%, n=10, P<0.05) in the neointimal area in the Epo-treated mice compared with the saline-injected group. The ratio of intima to medial area (I/M ratio) in the Epo-treated mice was significantly smaller (46%, n=10, P<0.05) than that in the saline-treated mice, whereas
medial thickness did not significantly differ between either group (data not shown).

As the involvement of NO in the neointimal hyperplasia has been reported, we next examined the effect of L-NAME on the Epo-mediated inhibition. Seven-day pretreatment with L-NAME significantly aggravated the neointimal hyperplasia in the saline-treated control mice (31%, n=10, P<0.05), consistent with the previous reports using eNOS-null mice. Interestingly, L-NAME completely abolished Epo-mediated inhibitory effect on the neointimal hyperplasia (I/M ratio: 1.61±0.38 versus 0.63±0.15 in the Epo-treat mice, P<0.05, n=10 each) (Figure 1A and 1B), suggesting the involvement of NO in Epo-mediated action. Furthermore, treatment with the NO donor L-arginine reduced the neointimal area to the level comparable to the Epo-treated mice (Figure 1A and 1B).

To confirm that the Epo-mediated inhibition of the neointimal formation is eNOS/NO dependent, we performed the arterial injury in the eNOS-null mice. The neointimal formation in the Epo-treated eNOS-null mice was aggravated (2.8-fold, n=7, P<0.005) compared with that of the Epo-treated wild-type mice, which was similar to the level in the Epo plus L-NAME–treated mice (2.9-fold, n=10, P<0.005) (Figure 1A and 1B).

The hemoglobin value was significantly higher after Epo treatment than that of the saline-treated controls (12.6±0.4 versus 14.0±0.1 g/dL at day 9, n=15, P<0.05). The number of white blood cells reached a peak on day 2 in both groups and was significantly higher in the Epo-treated mice (18 600±2020 versus 10 200±1100 cells/μL of control, n=15, P<0.05). The platelet number was similar in both groups on days 2 and 9.

**Epo Promotes Reendothelialization**

Evans blue dye was administered premortem to stain the nonendothelialized areas 5 and 14 days after injury. Nonendothelialized lesions are marked by blue staining, whereas the reendothelialized area appears white (Figure 2A). At both time points, the reendothelialized area in the Epo-treated group was significantly larger than that in the saline-treated group (1.4- and 1.8-fold on day 5 and -14, n=6, respectively) (Figure 2A). Immunostaining with anti-CD31 antibody in transverse sections revealed that the proportion of CD31+ endothelial area to the total lumen length in the Epo-treated mice was 65% lower than that in the eNOS-null–treated mice (n=10, P<0.01) and similar to the saline-treated control mice (Figure 2B).

**Epo Facilitates Mobilization of EPCs**

CD45dim cells were gated from PB MNCs and subsequently analyzed for the expression of endothelial lineage markers, Flk-1 and CD34. Three-day Epo treatment mobilized the CD45dim cells into circulation (Figure 3A), and the ratio of CD45dim/Flk-1+ cells to total PB MNCs increased to 7.4-fold (n=5 each, P<0.05) (Figure 3A, right). We further investi-
gated the effect of L-NAME pretreatment on the Epo-mediated mobilization of EPCs using anti-Sca1 and anti–Flk-1 antibodies. The number of circulating Sca1+/Flk-1+ cells in the control group was markedly increased than the basal level (2-fold, n=6, P<0.05), whereas in the L-NAME–treated group, the Epo-mediated mobilization was completely inhibited (Figure 3B). To confirm the endothelial property of CD45 dim/Flk-1+ cells, we cultured them for 7 days in EGM medium with 10 ng/mL VEGF, and the EC-specific function was examined. Confocal microscopy demonstrated that 93.5% of cultured CD45dim/Flk-1+ cells were bound to FITC-conjugated BS-1 lectin and incorporated DiI-labeled acLDL, a commonly used identifier of endothelial lineage cells (Figure 3C). To evaluate the number of circulating EPCs, PB MNCs were isolated 3 days after Epo or saline treatment and cultured for 7 days. The relative numbers of BS-1 lectin/+acLDL+ cells from Epo-treated mice were 5.2±0.5-fold higher (n=5, P<0.02) than that in the saline-injected control (Figure 3D).

Antiapoptotic Effect on Medial SMC After Vascular Wire Injury

The procedure of wire injury of mouse femoral artery causes complete removal of endothelium, resulting in rapid apoptosis of medial SMCs that enhances neointimal hyperplasia. Inhibition of this burst apoptosis is reported to be a preventive effect on neointimal hyperplasia. Interestingly, at 6 hours after injury, the number of apoptotic cell in the Epo-treated mice was increased to the level similar to the saline-treated mice, suggesting that Epo treatment did not prevent, but shifted, the apoptosis of VSMCs after wire injury.

The biological effects of Epo in the injured artery may be attributable to Epo-induced increase in circulating NO pool produced by the remote endothelial cells. Plasma NOX concentrations at 1, 2, 4, and 6 hours and 1, 3, and 14 days after injury of the Epo-treated mice were similar to those of the saline-treated controls, respectively. L-NAME treatment did not affect the Epo-mediated protection of VSMC apoptosis. Considering that the endothelium was completely removed by wire injury and the expression of EpoR in the EC-denuded artery was detected by RT-PCR analysis (unpublished observation, 2006), these findings suggest that Epo directly affects the apoptosis of VSMCs after wire injury. The expression levels for eNOS and EpoR in the lung and carotid artery at the same time points were comparable with the control mice when assessed by Western blotting and RT-PCR analysis, respectively (data not shown). These findings suggest that Epo-

Figure 2. Epo facilitated reendothelialization after wire injury. A, Evans blue dye was injected 10 minutes before euthanasia on days 5 and 14. Nonendothelialized lesions are marked by blue staining, whereas the reendothelialized area appears white (arrowhead). Quantification of the reendothelialized areas was performed with computed morphometry (n=6 each, *P<0.01, **P=0.02). B, Fourteen days after arterial injury with and without L-NAME pretreatment, the lesion was subjected to histological analysis. ECs were identified by immunostaining with anti-CD31 antibody in day-14 artery samples. Apparent CD31+ area is indicated by arrowheads. The ratio of CD31+ length to lumen perimeter in sections were evaluated and averaged on 5 different cross-sections from each artery (n=10, *P<0.05, **P<0.01). I indicates intima.
mediated action unlikely results from the nonspecific increase in circulating NO pool.

**Analysis by BM Replacement Model**

We further examined whether Epo actually induces the incorporation of marrow-derived EPCs into the regenerated endothelium by transplanting GFP-overexpressing or β-galactosidase BM cells to the BM-ablated background mice. Six weeks after transplantation, 96±2% and 94±3% of PB MNCs were replaced, respectively (n=5 each; FACS data not shown). The mice that received vascular injury were examined on day 14. GFP and β-Gal cells were patchily detected in the endothelial layer, and double-fluorescence immunohistochemistry (Figure 4A and 4B) disclosed a significant increase in the GFP+/CD31+ and β-Gal+/CD31+ lesions (2.7- and 2.2-fold, respectively, P<0.05) in Epo-treated mice compared with the saline-treated mice (Figure 4A). The ratio of Epo-induced GFP area in total CD31+ regenerated endothelium was 31±0.3% (n=5) (Figure 4A), suggesting that Epo-mediated reendothelialization is composed of differentiation of BM-derived EPCs as well as facilitated proliferation of resident ECs. In addition, the numbers of BM-derived macrophages (GFP+/MOMA2+) and vascular smooth muscle cells (GFP+/αSMA+) in the neointima of the Epo-treated mice were much lower than those of the control group (Figure 4A). GFP+/CD31+ or GFP+/B220+ cells were barely detectable in the neointima of both groups (data not shown).

**Expression of EpoR in Epo-Treated Injured Artery**

We investigated whether Epo-mobilized EPCs actually express the specific receptor for Epo (EpoR). EpoR-positive cells (brown staining, arrowheads in Figure 5A) were apparently localized on the regenerated endothelium in the Epo-treated arteries, whereas the expression of EpoR in the saline-treated injured or uninjured arteries was barely detectable. The immunofluorescence study using anti-EpoR, anti-CD31, anti-CD45, and anti–αSMA (αSMA) antibodies revealed that CD31+ endothelial cells in Epo-treated arteries coexpressed EpoR on the endothelial layer, whereas neither...
CD45− cells nor αSMA− cells expressed EpoR (Figure 5B). We also confirmed the expression of EpoR in the BM-derived EC-like cells mobilized by Epo using the mice repopulated with GFP BM cells (Figure 5C). Abundant GFP+/EpoR− cells were detected on the luminal surface in the Epo-treated injured artery, and the distribution was 2.5-fold greater than that in the saline-treated mice (P<0.05, Figure 5D).

**Epo Activates Akt-eNOS Pathways and NO Production in EPCs**

To investigate whether PB and BM EPCs express EpoR, Sca1−/Flk-1− EPCs were isolated from PB and BM by FACS and subjected to RT-PCR analysis. The DNA fragment corresponding to EpoR was amplified in the predicted size (Figure 6A). We further studied whether Sca1−/Flk-1− EPCs have the ability to activate the EpoR/Akt/eNOS pathway in response to Epo treatment. Because the PB volume obtained from the mice is too little to get the sufficient number of endothelial-lineage cells attaching on the plate, and also the attaching mice-derived cells are less spreading and less proliferative compared with the human cells, we used the human PB EPCs. The CD133+ EPCs were isolated from human PB by magnetic-associated cell sorting and primarily cultured. To study the stimulatory effect of Epo on the downstream pathway, after 12-hour serum starvation (0.5% serum), CD133+ EPCs were treated with 1.2 IU/mL Epo for 30 minutes. Double staining using anti-EpoR with anti–phosphorylated Akt or anti–phosphorylated eNOS antibodies showed that Epo markedly induced Akt-eNOS phosphorylation in EpoR-positive EPCs (Figure 6B).

We further measured the intracellular NO level with DAF-FM DA (10 μmol/L). NO was visualized as a green dot under laser microscopy (Figure 6C). Epo stimulation upregulated NO level to 1.7-fold higher than the untreated control level (P<0.05), whereas addition of l-NAME completely abolished this increase.

**Discussion**

The balloon-mediated injury of endothelial integrity stimulates a regeneration of the EC monolayer, but this regenerative process is slow and cannot prevent the early proliferative events leading to the onset of a neointimal lesion. A novel approach that promotes early reendothelialization is required to potentiate this natural regenerative process. In this study, we examined whether Epo treatment is a feasible strategy to cause reendothelialization of wire-injured vessels. Our results
demonstrated that the 3-day treatment of Epo increases the circulating Sca-1/VK-1 EPCs expressing an EpoR and that the mobilized EPCs contribute to the reendothelialization, leading to the inhibition of neointimal hyperplasia in an NO-dependent manner. Furthermore, we found that EpoR expression is upregulated in the Epo-induced neoendothelium and that the Epo/EpoR system causes the activation of the Akt/eNOS pathway on the EPCs and inhibits the apoptosis of medial SMCs.

EPCs can be harvested from PB, and intravenous transplantation of EPCs into EC-denuded vessels potentiates the recovery of endothelial integrity that causes the inhibition of neointimal hyperplasia, although cell transplantation protocols, such as the ex vivo expansion of EPCs, are technically challenging. G-CSF-induced EPCs were shown to enhance the repair of injured arteries and prevent intimal hyperplasia. However, it appears that the safety and feasibility of G-CSF treatment focusing on the induction of vascular occlusion in atherosclerotic lesions has not yet been established. In 12 intractable angina patients, the administration of G-CSF was associated with 2 cases of acute myocardial infarction and 1 case of cardiac death. There are articles reporting the induction of acute myocardial infarction and cerebral infarction in G-CSF–treated BM transplantation patients. Differentiation of G-CSF–mobilized progenitor cells into VSMC within the stented segment, induction of angiogenesis within the atherosclerotic lesion, and aggregation of mobilized inflammatory cells within the plaque may be plausible explanations.

This study showed that short-term (3-day) treatment with Epo after EC-denuded injury leads to accelerated reendothelialization and marked inhibition of neointimal formation. Figure 2A demonstrates that the ratio of reendothelialization by Epo and saline treatment is 73% and 41% on the total lumen area, respectively, whereas the coverage by CD31– marrow-derived cells on the reendothelialized lumen area is 75% and 32%, respectively (Figure 4B), indicating that 55% of Epo-mediated reendothelialized area is derived from marrow cells, and, in the saline-treated control, 13% of reendothelialized area is marrow-derived cells. Considering that Epo-mediated EPC mobilization (Figure 3) and reendothelialization (Figure 2) are NO dependent, it is suggested that EPC release via Epo and the endothelial differentiation in the repair process is partly involved in the observed reduction of neointima. Although the mobilization of EPC might be considered unfavorable for tumor growth, multiple clinical trials have recommended the use of Epo in chemotherapy-associated anemia or for longer survival among patients with multiple myeloma. Thus, the safety and feasibility of Epo treatment has been established. Although it was a concern that erythropoiesis may increase the risk of thrombosis because of an elevation in blood viscosity or cause hypertension resulting from the induction of the vasoconstricting hormone endothelin-1, thrombosis formation at the injured lesion in the Epo-treated group was similar to the saline-injected group, and blood pressure did not increase after short-term Epo-treatment (unpublished observation, 2005).

Moreover, Epo was reported to attenuate cytokine production and inflammation in tissue ischemia by targeting cell apoptosis or to induce cellular protection by activating Epo-EpoR signals involving Akt pathways, consistent with the present result (Figure 6). These anti-inflammatory and antiapoptotic cytoprotection actions associated with endothelial EpoR-mediated Akt/NO signaling may also contribute to the preventive effect of Epo on the neointimal hyperplasia.

EPC-like cells were reportedly derived from more differentiated CD34+ or immature CD133+ hematopoietic stem cells, as well as from PB MNCs or CD14+ monocytes. These EPC-like cells lose CD45 hematopoietic markers and express endothelial markers. Our present study clearly showed that CD45/VK-1 or Sca1/VK-1 EPCs were mobilized by Epo, had a functional EC-like property in vitro, and contributed to endothelial regeneration after wire-mediated injury, suggesting this cell type is an EPC-like cell responsible for Epo-induced vascular repair. Thus, Epo treatment may be a novel strategy to inhibit the neointimal hyperplasia by directly acting on the injured vessels as well as mobilizing EPCs to the neoendothelium.
Figure 6. Epo-mediated activation of Akt/eNOS and NO production in PB-derived endothelial progenitor cells. A, The Sca1+/Flik-1- EPCs were isolated from the PB and BM by FACs. Total RNAs were prepared from them and the whole BM cells and subsequently subjected to RT-PCR analysis using the primers for EpoR and GAPDH. The DNA fragments corresponding to EpoR and GAPDH were amplified in the predicted sizes. B, CD133+ progenitor cells were purified to >90% purity from human PB MNCs by positive selection using anti-CD133+ microbeads and a magnetic cell sorting device and then were primary cultured. After 12 hours of serum starvation (0.5% serum), they were stimulated by 1.2 IU/mL Epo for 30 minutes and immunostained for EpoR (green) with phosphorylated Akt (red) or phosphorylated eNOS (red) (bar=25 μm). Double-fluorescence–positive cells appear yellow (arrowheads). C, Measurement of the intracellular NO level. The CD133+ cells were loaded with DAF-FM DA (10 μmol/L), and NO was visualized as a green dot under laser microscopy. The averaged intensity in the Epo and the Epo plus L-NAME cells relative to the control group was evaluated. *P<0.005 (n=8, each).

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References


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