Role of erythropoietin for angiogenesis and vasculogenesis: from embryonic development through adulthood


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Müller-Ehmsen, Jochen, Annette Schmidt, Benjamin Krausgrill, Robert H. G. Schwinger, and Wilhelm Bloch. Role of erythropoietin for angiogenesis and vasculogenesis: from embryonic development through adulthood. Am J Physiol Heart Circ Physiol 290: H331–H340, 2006. First published July 15, 2005; doi:10.1152/ajpheart.01269.2004.—Erythropoietin (EPO), a stimulator of erythropoiesis, was previously shown to stimulate angiogenesis and proliferation of endothelial cells. Here, we investigated and compared the influence of EPO on cell number, proliferation, apoptosis, migration, and differentiation of endothelial cells in intact mouse embryoid bodies (EB), isolated endothelial cells from EB (EBEC), and adult human endothelial progenitor cells (hEPC). EB were treated with EPO (0.5 U/ml) immediately after plating was completed (day 5+0) or 3 days later. EPO treatment was continued until days 5+3 or 5+6. Cultured EBEC were treated 3 days after being plated, and primary hEPC from young healthy adults were treated 5 days after being plated with EPO for 48 h. Immunohistochemistry was performed with anti-PECAM (CD31), anti-Ki67, anti-CD34, anti-CD133, anti-EphB4, and anti-ephrinB2 antibodies. In all, mouse EB and EBEC and hEPC, EPO-treatment resulted in increased number of endothelial cells, increased proliferation, decreased apoptosis, and enhanced migration. In EB, this EPO effect was most pronounced when treatment was begun early (day 5+0) and was accompanied by an enhanced endothelial tube formation. In EBEC and hEPC, EPO shifted the phenotypic differentiation toward an increased ratio of EphB4-positive cells, i.e., toward a venous phenotype. These results are consistent with an important role of EPO for the number, proliferation, apoptosis, function, and phenotypical development of immature endothelial cells, which persists from early development through adulthood. They provide additional and further evidence for a strong interrelation between hematopoiesis and vasculogenesis/angiogenesis (sharing the same pathways), which may be important in many physiological and pathophysiological conditions.

embryoid body; endothelial cells; endothelial progenitor cells; monocellular cells

THE RENAL GLYCOPROTEIN ERYTHROPOIETIN (EPO) is a cytokine that stimulates erythropoiesis and erythrocYTE differentiation. Its presence is crucial for normal development and survival, whereas the absence of EPO or EPO receptors leads to reduced primitive erythropoiesis and death during embryogenesis (21). EPO receptors are also expressed in mature endothelial cells (1), and, consequently, EPO has been shown to activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT)- and the phosphatidylinositol 3-kinase (PI3K)/Akt pathways, which elicit a proliferative, promigratory, and anti-apoptotic effect in these cells (6, 12, 14). In cultured neonatal microvascular endothelial cells, EPO was found to stimulate proliferation and tube formation (2). Recently, additional effects of EPO within the cardiovascular system have been discovered, including support of cardiac morphogenesis in embryonic development (20) and cardioprotection from ischemic (13) or ischemia-reperfusion injury (4). In addition, recent studies provided evidence that EPO may also play a role in vascular repair in adult animals. In mice treated with EPO, the number of endothelial progenitor cells (EPC) was significantly increased in the bone marrow and in peripheral blood. After ligation of the superficial and deep femoral artery, neovascularization of the hindlimb was significantly enhanced by EPO treatment (9). In patients with coronary artery disease, the number and function of circulating EPC were closely related to serum levels of EPO (9). In accordance with this observation, patients with renal failure, and therefore reduced EPO levels, have lower number of endothelial progenitor cells in peripheral blood than healthy control subjects. Treatment with EPO restored that number, and when healthy control subjects were treated with EPO, a “superphysiological” increase in the EPC number was observed (3). Thus in mature endothelial cells, EPO is known to directly enhance proliferation and migration and to reduce apoptosis (15). In addition, treatment of adult mice and men with EPO seems to increase the number of circulating EPC in peripheral blood. However, the immediate cellular response of isolated immature endothelial cells on stimulation with EPO is not known.

Angiogenesis and vasculogenesis are crucial features of embryonic development and endogeneous response to hypoxia, and their enhancement may serve as putative future therapy for ischemic diseases such as ischemic heart disease in the adult (11). For the development of such therapies it is important to understand whether the mechanisms and signal transduction pathways that play a role for vessel formation are preserved during development and maturation. Therefore, in the present study, the effects of EPO on cells at different stages of development, i.e., embryoid bodies (EB), endothelial cells isolated from EB (EBEC), and human EPC (hEPC) from adult volunteers, were compared. Mainly, our results show that EPO directly increases the number, proliferation, and migration of endothelial cells in or isolated from EB and adult endothelial progenitor cells, while apoptosis is reduced. As a result, tube formation was enhanced in EB. In cultured EBEC and hEPC a venous phenotype was propagated, providing evidence for a role of EPO for phenotypic differentiation. Thus EPO may play...
a role for vasculogenesis and angiogenesis from early development through adulthood.

MATERIALS AND METHODS

Mouse blastocyst-derived embryonic stem (ES) cells were established and maintained in culture as described previously (7). ES cells were cultured on a fibroblast feeder layer in DMEM, supplemented with 15% heat-inactivated fetal calf serum (FCS), 50 μU/ml penicillin, 50 μU/ml streptomycin, 200 μM L-glutamine, 100 μM β-mercaptoethanol, and 1% MEM (GIBCO-BRL, Gaithersburg, MD). They were cultured for differentiation in hanging drops, as described previously (8). Six hundred cells were cultured in 20 μL of DMEM, supplemented with 20% FCS (plus additives like mentioned above), hanging from the lid of the culture dish for 2 days, allowing formation of cell aggregates (EB). Subsequently, the aggregates were incubated for 3 days in suspension and plated in a 24-multwell plate on gelatin-coated coverslips for 12 days (15% FCS). After treatment with EPO, cells were fixed with 4% paraformaldehyde in 0.1 M PBS for 25 min and washed with 0.1 M PBS.

Isolation, magnet-associated cell sorting, and cultivation of EBEC.

EB, cultured as described above, at the age of 5+7 days were dissociated with Accutase (PAAM Laboratories, Linz, Austria). The single cell solution was washed with medium and incubated with an endothelium-specific marker rat anti-mouse-PECAM-1 (CD31) (1:800, monoclonal antibody, Pharmingen, San Diego, CA) for 30 min at 37°C, 5% CO2. Cells were washed with magnet-associated cell sorting (MACS) buffer (0.5% BSA and 2 mM EDTA in PBS) and incubated with the secondary antibody, which is conjugated with beads goat anti-rat IgG microbeads (1:4, Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min at 6–8°C. After being washed, cells were MACS sorted by using a mini-MACS system with MS separation columns (Miltenyi Biotec). The positive fraction was cultured on gelatin-coated dishes. After 2–3 wk the endothelial cells were passaged for the first time and afterwards once a week. At passage 3 cultured MACS-sorted endothelial cells were plated and 3 days later were incubated with and without EPO for 48 h and were stained for immunohistochemistry as described below. The purity of EBEC amounted to 89% as tested with low-density lipoprotein labeled with dioctadecyl-3,3,3’-tetramethylindo-carbocyanine perchlorate (DiI-Ac-LDL, Paesel, Lorei, Hanau, Germany) and different endothelium-specific markers (flk-1, flt-1, VE-Cadherin, CD31).

Isolation and culture of hEPC from peripheral blood. Whole blood samples were obtained from healthy volunteers and collected in EDTA-enriched containers. Mononuclear cells were separated by gradient centrifugation (lymphocyte separation medium, PAA Laboratories) at 800 g for 30 min. The layer containing mononuclear cells was collected and washed twice with PBS. The mononuclear cells (still containing hematopoietic cells) were then plated on fibronectin-coated dishes (1 × 106 cells/cm2) and incubated with endothelial cell growth medium MV2 (PromoCell, Heidelberg, Germany). The medium was changed, and the nonadherent cells were discarded after 3 days. At day 5 the adherent cells (endothelial progenitor cells) were used for further experiments. The purity of EPC amounted to 93 ± 3% as tested with DiAc-5LDL (Paesel + Lorei) and lectin and different endothelium-specific markers (flk-1, flt-1, VE-Cadherin, CD31).

EPO treatment.
The concentration of EPO (Boehringer Mannheim) was used was 0.5 U/ml based on previously published observations (14). EBs were treated with EPO under three different conditions and compared with control (no EPO): One group was treated from the day of plating (day 5+0) for 3 days (until day 5+3); one group was treated from the day of plating (5+0) for 6 days (until day 5+6); and one group was treated beginning at day 3 after being plated (day 5+3) until day 6 after being plated (day 5+6). The EBEC and the hEPCs were treated with EPO for 48 h 3 days after being plated or at day 5 after being plated, respectively. All analyses were done immediately after EPO treatment.

Immunohistochemistry and assessment of cell numbers. Cells were fixed with 4% paraformaldehyde (in 0.1 M PBS), washed several times with 0.05 M TBS, and subjected to 0.25% Triton X-100 and 0.5 M NH4Cl in 0.05 M TBS, for cell membrane destruction. For blocking probes, 5% BSA in Tris-buffered saline were used (1 h, room temperature). The primary antibodies were diluted in 0.8% BSA and incubated overnight at 4°C. As primary antibodies anti-mouse-PECAM (1:800, rat, Pharmingen, San Diego, CA), anti-human PECAM (1:300, mouse, Biogenex, San Ramon, CA), ephrinB2 (1:400, rabbit, Santa Cruz, Heidelberg, Germany), EphB4 (1:400, rabbit, Santa Cruz), CD34 (1:300, rabbit, Santa Cruz), CD133 (1:100, mouse, Miltenyi) were used. Secondary antibodies, sheep anti-rat IgG biotinylated (1:400, Amersham, Life Science, Little Chalfont, Buckinghamshire, UK), goat anti-rabbit Ig conjugated Cy2, goat anti-mouse Ig conjugated Cy2 (1:200, Dianova, Hamburg, Germany, absorption 495 nm, emission 519 nm), as well as streptavidin-conjugated Cy3 (1:500, Amersham, Life Science, absorption 552 nm, emission 565 nm) were used for cells. The incubation with secondary antibody was done in TBS for 1 h at room temperature in the dark. For negative control, experiments were done with secondary antibody only (see insets in Figs. 1, 2, 4, and 5). For microscopy an Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) was used for light and fluorescence (Cy3-filter: Zeiss 510–560 FT580 LP (No. 14), Cy2-filter: AHF HQ-filterset for Cy2 sel, emitter HQ 527/30, beam-splitter Q 505LP, exciter HQ 480/40). The numbers of EBEC and the numbers of hEPC were assessed by counting 10 fields of view in a ×400 magnification (each n = 4 independent experiments). The numbers of CD31/CD133 and CD31/CD34 double-positive cells were analyzed from randomly chosen 50 vessel-like tubes (each n = 4).

Proliferation. The influence of EPO on proliferation of vascular cells in EB, cultured MACS-sorted EBEC, and hEPCs was analyzed by treating EBs and cells with and without EPO. EBs and cells were fixed and used for immunohistochemistry as described above. After endothelial cells were identified by immunohistochemistry, proliferating cells were detected with the primary rabbit anti-Ki67 antibody (polyclonal, 1:150, Dianova). For secondary antibody, we used goat anti-rabbit conjugated CY2 (1:200, Dianova). The numbers of Ki67-positive cells within 50 randomly chosen vessel-like tubes (EB) or in 10 fields of view (×400 magnification, cell culture) were counted as proliferating cells as described previously (16). For each condition four independent experiments were performed.

Apoptosis. The effect of EPO on endothelial cell apoptosis was investigated in EBEC and hEPC as described before (16). In brief, after fixation the apoptotic cells were detected with a rabbit anti-PARP p85 fragment antibody (polyclonal, 1:250, Promega, Madison, WI). As a secondary antibody, goat anti-rabbit conjugated CY2 was used. Ten fields of view were analyzed (×400 magnification), and the poly(ADP-ribose) polymerase (PARP)-positive cells were counted as apoptotic cells.

Migration assays. Migration assays were performed in a modified Boyden-Chamber by using a 24-well HTS FluoroBlok insert system (Falcon Becton Dickinson, Heidelberg, Germany). The inserts contain a membrane out of polyethylene with 8.0-µm pores, which block >99% of the light transmission in a wavelength region between 490 and 700 nm. Single cells (104) were placed on top of an insert and incubated for 8 h in their specific medium (see above) for control or additionally with EPO. Cells were fixed with 4% paraformaldehyde in 0.1 M PBS. The membrane was then cut out of the insert and covered in 4′,6-diamidino-2-phenylindole mounting medium (absorption 360 nm, emission 460 nm; Vectashield, Vector Laboratories, Burlingame, CA); between two thin coverslips. The total number of migrated cells was counted (n = 4).

Statistical analysis. All data are presented as means (SD). Data analysis were performed by using analysis of variance with Bonferroni...
roni post hoc test and/or Student’s t-test for unpaired data. Significance was set at a $P$ value < 0.05.

RESULTS

Cell number in response to EPO treatment. Treatment with EPO increased the number of PECAM-positive cells (endothelial cells and angioblasts) in EB (Fig. 1, A–D) and cultured MACS-sorted EBEC (Fig. 1, E and F). In EBs this effect was most pronounced when treatment was begun at day 5+0 with no difference for the duration of treatment (until day 5+3, Fig. 1D, vs. day 5+6, Fig. 1C). When treatment was begun later after EB formation (at day 5+3), the effect was abolished (Fig. 1B). A fourfold increase in cells carrying the endothelial cell marker protein PECAM was observed after treatment of EBEC with EPO for 48 h (4,112 ± 623 cells) versus control (1,051 ± 307 cells) ($P < 0.001$; Fig. 1, E and F; see Fig. 1G for quantitative data). Also, the number of adult hEPC was increased by treatment with EPO (101 ± 11 cells) for 48 h compared with control (24 ± 5 cells) ($P < 0.001$; Fig. 1H).

Proliferation, apoptosis, and migratory activity of vascular cells in EBs, EBEC, and hEPC in response to EPO. To analyze the mechanisms by which the number of endothelial cells is increased, the influence of EPO on proliferation and apoptosis was assessed. Furthermore, the functional impact of EPO treatment on EBEC and hEPCs was tested with a cell migration assay. Cell proliferation was assessed by immunostaining with

![Fig. 1. Increase in the number of endothelial cells in response to EPO (EPO).](image-url)

Embryoid bodies (EB) (A–D) and cultured magnet-associated cell sorting (MACS) sorted endothelial cells from EB (EBEC) (E and F) were incubated with erythropoietin (EPO, 0.5 U/ml) for 48 h. Immediately after treatment, tissue and cells were stained with a rat anti-mouse PECAM antibody visualizing endothelial cells, angioblasts, and vessel-like tubes. White bars in A–D depict the pattern of EPO treatment: control (A), treatment from day 3 after plating until day 6 after plating (B), from day of plating until day 6 after plating (C) and from day of plating until day 3 of plating (D). EBEC (E–G) and human endothelial progenitor cells (hEPC) (H) were treated for 48 h. In EB, angioblasts and vessel-like tubes were seen more frequently in the groups that were treated from day 0 (C and D) compared with control (A) or the group in which treatment was begun at day 3 (B). EBEC were increased in number after 48 h of EPO treatment (F) compared with untreated control (E); for quantitative analysis see G. Similarly, EPO increased the number of hEPCs (H) (both $*P < 0.001$ vs. control). Inset in A: negative control in EB with secondary antibody only. Inset in E: negative control in EBEC with secondary antibody only.
Ki67 antibody. In EBs, endothelial cell proliferation was enhanced on treatment with EPO (day 5+0 for 6 days: 871 ± 117 vs. 137 ± 11 cells; *P* < 0.001) (Fig. 2, C and E). The effect was similarly pronounced when treatment was stopped after 3 days (day 5+0 until day 5+3; 947 ± 34 cells; *P* < 0.001) (Fig. 2, D and E). Also, when treatment with EPO was not started before day 5+3 (and continued until day 5+6), proliferation was increased over control (592 ± 57, *P* < 0.001) (Fig. 2, B and E). However, this number was lower than that observed at early exposure to EPO (from day 5+0, *P* < 0.001) (Fig. 2, A and E). EBEC also responded to EPO treatment with increased proliferation (EPO: 199 ± 32 cells; control: 69 ± 16 cells; *P* < 0.001) (Fig. 2F). For adult hEPC a trend toward increased proliferation was observed on EPO treatment without reaching statistical significance (EPO: 54 ± 26 cells; control: 37 ± 9 cells) (Fig. 2G).

Apoptotic cells were quantified in EBEC and in adult hEPC by detection of PARP p85 fragment. Apoptotic events were reduced in EBEC by the treatment with EPO (EPO: 17 ± 4 cells; control: 26 ± 4 cells; *P* = 0.011) by ~35% (Fig. 3A). In hEPC, a slight but nonsignificant reduction in apoptosis was observed (EPO: 15 ± 4 cells; control: 18 ± 5 cells) (Fig. 3B). However, as for migration, both EBEC (EPO: 1,348 ± 82 cells; control: 762 ± 107 cells; *P* < 0.001) and adult hEPC (EPO: 141 ± 20 cells; control: 97 ± 4 cells; *P* = 0.005) were significantly activated by EPO.
The number of CD31 and CD34 double-positive cells relative to all CD31-positive cells was also observed in EBEC (CD31/CD133: EPO 107 ± 24 cells; control 42 ± 10 cells; P = 0.007; CD31/CD34: EPO 230 ± 30 cells; control 86 ± 8 cells; P = 0.001) (Fig. 6B) and in adult hEPC (CD31/CD133: EPO 26 ± 7 cells; control 12 ± 3 cells; P = 0.01; CD31/CD34: EPO 31 ± 8 cells; control: 15 ± 3 cells; P = 0.023) (Fig. 6C).

Arterial versus venous differentiation of EBEC and of hEPC in response to EPO. Anti-EphB4 and anti-ephrinB2 antibodies were used to distinguish the venous versus the arterial phenotype of endothelial cells in response to EPO treatment (48 h) of EBEC and adult hEPC. In both, EBEC and EPC, we found that the ratio of EphB4-positive cells versus ephrinB2-positive cells was increased, i.e., the ratio of venous versus arterial phenotype increased on EPO treatment (Fig. 7, A and B, for hEPC). For EBEC the ratio EphB4 to ephrinB2 was 1.11 ± 0.37 for control and 1.29 ± 0.11 for EPO-treated cells (P = 0.005, Fig. 7C). For hEPCs the ratio EphB4 to ephrinB2 was 1.17 ± 0.12 for control and 1.27 ± 0.53 for EPO-treated cells (P = 0.001, Fig. 7D).

**DISCUSSION**

Previous studies have suggested a role for EPO in vasculogenesis and angiogenesis (2, 9, 14). Whereas the induction of cell proliferation and migration and the reduction of apoptosis have been well described in mature endothelial cells (15), for immature endothelial cells the available information is limited. Furthermore, for the understanding of angiogenesis and vasculogenesis and for the future development of angiogenic therapies, it is important to know whether the same pathways are active at different stages of development. Therefore, we investigated EPO effects on number, proliferation, apoptosis, migration, and differentiation of endothelial cells in mouse EBs, EBEC, and adult hEPC. Before this study we were able to detect EPO receptors on the surfaces of all cell types used in the present study (data not shown), which is an obvious prerequisite for any specific effect of EPO.

In EBs, previous studies have shown that a mixture of growth factors (erythropoietin, interleukin-6, fibroblast growth factor 2, and VEGF) increases vessel formation (18). Here, we find that addition of EPO alone is capable to do so. Therefore, EPO may play a fundamental role in vascular development of the embryo. The effects of EPO are mediated by an increased number of endothelial (PECAM positive) cells that were found within the embryoid body and after cultured EBECs were treated with EPO. The increased number of endothelial cells is at least in part accomplished by an enhancement of prolifera-

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**Fig. 3.** Decrease in apoptosis and increase in migratory activity of endothelial cells in response to EPO treatment. Apoptosis of cultured MACS-sorted EBEC is significantly reduced after the incubation with EPO (A) (P = 0.011). In contrast, apoptosis of hEPCs that were isolated from peripheral blood of adult healthy volunteers was not significantly influenced by EPO (B). On the other hand, migration is enhanced in both EBEC (C) and hEPC (D) (P < 0.001 and P = 0.005 vs. control, respectively).
tion and a reduction of apoptosis. Furthermore, a more activated state of the cells was observed after EPO treatment, as indicated by a higher migratory activity. Finally, the percentage of cells with progenitor cell markers (CD34 and CD133) was increased within the endothelial cell population, indicating a preferential activation of endothelial precursors by EPO. Interestingly, treatment of EB from day 5+0 was most effective to increase vasculogenesis, whereas treatment that was

Fig. 4. Increase in CD34-positive cells in EB as a response to treatment with EPO. EB were treated with EPO day 0 to day 6 (E and F), day 3 to day 6 (C and D), or day 0 to day 3 (G and H). Immunofluorescence was used to visualize the cells that were both CD31 (PECAM) positive (A, C, E, G) and CD34 positive (B, D, F, H). Compared with control (A and B), all EPO-treated EBs had an increased number in cells that stained positive for both CD31 and CD34. These cells may represent progenitors of both endothelial cells and hematopoietic cells (hemangioblasts?). Fifty vessel-like tubes were analyzed. Insets in A and B: control experiments with secondary antibody only.
begun at day 5+3 had only marginal effect. Thus the EPO effect appears highly regulated with either the expression of EPO-receptor being turned off during development and/or with the target cells (hemangioblasts) being present in larger number at day 5+0, whereas their number is reduced during EB development (5).

Interestingly, similar effects as in day 5+0 EB and their cultured endothelial cells were observed in adult hEPC (cell number and migration were increased, proliferation showed a trend toward increase, apoptosis showed a trend toward decrease, and a higher percentage of immature cells was found).

This finding suggests that although the effect of EPO on immature endothelial cells may be markedly reduced when EB are exposed at day 5+3, the stimulatory response is reestablished (or still preserved) in hEPC from adults. This finding offers an interesting cellular biological mechanism for the increased number and functional capacity (neovascularization) of EPC seen in mice and men previously (3, 9); at least one part of the observed EPO effects on EPC can be attributed to enhanced proliferation and reduction in apoptosis. Whether the proliferation takes place in the circulation or within the bone marrow remains unclear and will need to be addressed in
future studies. Thus it is possible that on EPO stimulation the number of EPC is increased by proliferation in the peripheral blood rather than by mobilization from the bone marrow. Similar as in EBEC we found that more hEPC expressed markers of immaturity, supporting again the preferential stimulation of immature cells by EPO. The cells that stained double positive for CD31 and CD34 and/or the cells that stained double positive for CD31 and CD133 may represent in part the cell population that contains hemangioblasts (5).

With respect to venous versus arterial differentiation of the cells, the number of endothelial cells carrying markers for venous determination increased on EPO treatment (for both EPC and EBEC). The influence of EPO on phenotypic differentiation of the endothelial cells shows a further kind of regulation of arterial or venous phenotype of endothelial cells, besides the recently shown flow-dependent regulation of ephrinB2 during embryonic vascular development (10). Considering the important role of ephrinB2 and EphB4 for the development of a functional vascular network (19), the role of EPO for the development of functional vascular networks should be investigated in further studies. The interaction of EphB4 (erythroid cells) and ephrinB2 (bone marrow stromal cells) is also important for the maturation of erythroid cells in bone marrow and, thus, for bone marrow erythropoiesis (17), further supporting the similarity of pathways for the regulation of hematopoiesis and angiogenesis. For the findings of the present study, it needs to be noted that an artificial system of endothelial cell culture was studied and that the impact of EPO on the differentiation of the cells may differ in vivo under the influence of shear stress and other factors.

In summary, the present study supports a relevant impact of EPO, known as a stimulator of erythropoiesis, on the number, proliferation, apoptosis, and function of immature endothelial cells. The EPO effects and the EPO pathways are present in embryonic vasculogenesis and are reestablished (or persist) in endothelial progenitor cells of adult humans. Apparently a venous type differentiation is supported, indicating an additional role of EPO for phenotypic differentiation. Together with previous publications, our data support the notion that there are common progenitors and common regulatory pathways of vasculature and the hematopoietic system. These pathways seem to persist from early development through adulthood and suggest a persistent strong interrelation between hematopoiesis and vasculogenesis/angiogenesis.

As a limitation it is noteworthy that these data do not answer the question whether or not the observed effects could be provoked by stimulation with other growth factors as well.

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**Fig. 6.** CD31/CD34 (left) and CD31/CD133 (right) double-positive cells in response to EPO treatment. Both CD31/CD34- and CD31/CD133-positive cells were increased in vessel-like tubes from EB (A), in cultured EBEC (B), and in adult hEPCs (C) (all P < 0.05). Cells were counted from 50 vessel-like tubes or 10 fields of view, ×400 magnification, respectively.
addition, it cannot be excluded that the role of EPO in concert with other growth factors may be different from our observations with EPO alone.

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