

Impaired *in vivo* vasculogenic potential of endothelial progenitor cells in comparison to human umbilical vein endothelial cells in a spheroid-based implantation model

G. Finkenzeller*, S. Graner*, C. J. Kirkpatrick†, S. Fuchs† and G. B. Stark*

*Department of Plastic and Hand Surgery, University of Freiburg Medical Center, Freiburg, Germany, and †Institute of Pathology, Johannes Gutenberg University, Mainz, Germany

Received 29 April 2008; revision accepted 4 August 2008

Abstract

Objectives: Neovascularization represents a major challenge in tissue engineering applications since implantation of voluminous grafts without sufficient vascularity results in hypoxic cell death of implanted cells. An attractive therapeutic approach to overcome this is based on co-implantation of endothelial cells to create vascular networks. We have investigated the potential of human endothelial progenitor cells (EPC) to form functional blood vessels *in vivo* in direct comparison to vascular-derived endothelial cells, represented by human umbilical vein endothelial cells (HUVEC).

Materials and methods: EPCs were isolated from human peripheral blood, expanded *in vitro* and analysed *in vitro* for phenotypical and functional parameters. *In vivo* vasculogenic potential of EPCs and HUVECs was evaluated in a xenograft model where spheroidal endothelial aggregates were implanted subcutaneously into immunodeficient mice.

Results: EPCs were indistinguishable from HUVECs in terms of expression of classical endothelial markers CD31, von Willebrand factor, VE-cadherin and vascular endothelial growth factor-R2, and in their ability to endocytose acetylated low-density lipoprotein. Moreover, EPCs and HUVECs displayed almost identical angiogenic potential *in vitro*, as assessed by *in vitro* Matrigel sprouting assay. However *in vivo*, a striking and unexpected difference

between EPCs and HUVECs was detected. Whereas implanted HUVEC spheroids gave rise to formation of a stable network of perfused microvessels, implanted EPC spheroids showed significantly impaired ability to form vascular structures under identical experimental conditions.

Conclusion: Our results indicate that vascular-derived endothelial cells, such as HUVECs are superior to EPCs in terms of promoting *in vivo* vascularization of engineered tissues.

Introduction

Tissue vascularization is one of the major challenges to be addressed for clinical success of tissue engineering applications. Without rapid and high level of vascularization of transplanted grafts, the majority of cells fail to survive the early post-transplantational phase (1). Amongst therapeutic approaches to overcome the problem of insufficient vascularization are use of angiogenic growth factors embedded into appropriate scaffolds to promote ingrowth of microvessels (2–4), and cell-based concepts to enhance vasculogenesis (5,6). Using Bcl-2-transduced human umbilical vein endothelial cells (HUVEC), microvascular networks in collagen-fibronectin gels have been formed within 4 weeks of implantation into immunodeficient mice (5). Similar results have been reported with HUVECs co-implanted with mesenchymal precursor cells, which led to formation of functional, long-lasting microvessels upon implantation into immunocompromised mice (6). However, this effect was strictly dependent on co-seeding of mesenchymal precursor cells having the potential to differentiate *in vivo* into mural cells, suggesting that stabilization of newly formed vessels by mural cells represents a critical step in generating stable vasculature. We have recently reported the development of a spheroid-based endothelial cell transplantation technique for formation of

This work was performed at the Department of Plastic and Hand Surgery, University of Freiburg Medical Center, Freiburg, Germany.

Correspondence: G. Finkenzeller, Department of Plastic and Hand Surgery, University of Freiburg Medical Center, Hugstetter Str. 55, D-79106 Freiburg, Germany. Tel.: + 49 (0)761 270 6367; Fax: + 49 (0)761 270 6368; E-mail: guenter.finkenzeller@uniklinik-freiburg.de

durable and perfused blood vessels *in vivo* (7). In this model, Matrigel/fibrin-immobilized spheroidal HUVECs were able to form a network of stable and perfused blood vessels in mice without the need of co-seeding mural cells.

Endothelial progenitor cells (EPC) represent a very interesting alternative cell source for mature endothelial cells in tissue engineering applications. EPCs can be found in peripheral blood as well as in the bone marrow (8), from which they can be mobilized by action of various growth factors and cytokines (9,10). EPCs have already been used as a cell source for enhancing angiogenesis in the field of regenerative medicine, and have been proven to be effective in regenerating infarcted myocardium by supporting neovascularization (11,12).

In the present study, we investigated whether human EPCs would have the potential to form functional blood vessels *in vivo*. To test this, we analysed *in vivo* vasculogenic potential of EPCs in direct comparison to vascular-derived endothelial cells (HUVECs), in a xenograft model where human spheroidal endothelial aggregates were mixed in a Matrigel/fibrin matrix and implanted subcutaneously into SCID mice. Whereas implanted HUVEC spheroids gave rise to complex and stable three-dimensional networks of perfused human neovessels, which matured by recruiting mouse mural cells, implanted EPC spheroids showed significantly impaired ability to form blood vessels *in vivo*. Moreover, the few vessels derived upon EPC spheroid implantation were not able to recruit mouse perivascular cells and were not perfused.

Although HUVECs and EPCs have almost identical phenotypical and functional properties *in vitro*, *in vivo* data clearly demonstrate an important difference between vascular-derived endothelial cells and EPCs regarding their *in vivo* vasculogenic potential.

Materials and methods

Isolation of human EPCs and cell culture

Human EPCs from five different donors were isolated and expanded as previously described (13,14). In brief, peripheral blood mononuclear cells were isolated from 50 ml of peripheral blood from human volunteers or from human blood-derived buffy coats, with slight modification by density gradient centrifugation with biocoll separation solution (Biochrom, Berlin, Germany). Cells from individual volunteers were plated in 25-cm² culture flasks coated with rat type I collagen (50 µg/ml) in EGM-2 (Lonza, Cologne, Germany) with supplement mix (human epidermal growth factor, vascular endothelial growth factor (VEGF), human fibroblast growth factor-B, R3-insulin-like growth factor-1, ascorbic acid, heparin, gentamicin/amphotericin-B) and 10% foetal calf serum (FCS), and were cultured at 37 °C

with 5% CO₂, in a humidified atmosphere. After 4 days in culture, non-adherent cells were removed by washing with phosphate-buffered saline (PBS), new media were applied, and cultures were maintained for another 4–5 weeks. Media were changed every 3 days. After reaching approximately 80% confluence, cells were trypsinized then seeded into 75-cm² culture flasks, coated with human fibronectin (50 µg/ml) (Sigma, Deisenhofen, Germany) for expansion. EPCs from passages 2–5 were used for experiments.

HUVECs were purchased from Promocell (Heidelberg, Germany) and were cultured in endothelial cell growth medium (ECGM, Promocell) supplemented with 10% FCS and supplement mix (epidermal growth factor, hydrocortisone, basic fibroblast growth factor (bFGF)) at 37 °C and 5% CO₂ in 75-cm² tissue culture flasks. HUVECs from passages 2 to 5 were used for experiments.

Cell staining

For acetylated low-density lipoprotein (acLDL) uptake experiments, adherent cells were incubated in EGM-2 (Lonza, Cologne, Germany) supplemented with single aliquots and 10% FCS with 20 µg/ml (Dil)-labelled acLDL (Invitrogen, Karlsruhe, Germany) for 4 h at 37 °C in 5% CO₂ in a humidified atmosphere. After washing with PBS, cells were fixed with 2% paraformaldehyde for 10 min at 37 °C. After fluorescence staining, intensity was measured using an inverted fluorescence microscope using the rhodamine filter setting.

For immunostaining, cells were fixed in methanol and were incubated for 30 min with blocking solution (5% goat serum, Sigma) followed by incubation with the respective primary antibodies, monoclonal mouse anti-CD31 antibody (diluted 1 : 25, Dako, Hamburg, Germany), monoclonal mouse anti-VEGF-R2 antibody (antibody 3G2, diluted 1 : 200) (15), monoclonal mouse anti-VE-cadherin antibody (diluted 1 : 100, Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-CD45 antibody FITC-conjugated (diluted 1 : 100, Becton Dickinson, Heidelberg, Germany), monoclonal mouse anti-CD14 antibody FITC-conjugated (diluted 1 : 100, Becton Dickinson) and monoclonal mouse anti-von Willebrand factor antibody (diluted 1 : 200, Dako), for 1 h at room temperature. After washing with PBS, corresponding secondary antibodies (ready-to-use horseradish peroxidase-conjugated goat anti-mouse immunoglobulin; Dako) were applied and incubated for another 30 min. Thereafter, cells were exposed to AEC chromogen substrate (ready-to-use, Dako) and were weakly counterstained with haematoxylin. For FITC-fluorescence detection, cells were photographed using an inverted fluorescence microscope.

For histochemical examination of *in vivo* blood vessel formation, Matrigel/fibrin implants were removed 3 weeks

after xenografting, fixed in buffered formalin for 24 h, embedded in paraffin wax and sectioned at 5 µm. Sections were stained with haematoxylin and eosin (H&E) and examined for presence of luminal structures. Immunohistochemical analyses were performed on deparaffinised, rehydrated sections. Sections for immunoperoxidase staining were treated with 3% H₂O₂ to inhibit activity of endogenous peroxidase. After washing in PBS, sections were incubated for 30 min with blocking solution (goat serum, ready-to-use, Zymed, San Francisco, CA, USA) followed by incubation with corresponding mouse monoclonal primary antibodies: anti-human CD34 (diluted 1 : 75, Novocastra, Newcastle upon Tyne, UK) or anti-human CD31, (diluted 1 : 25, Dako) in a humidity chamber at room temperature for 1 h. For immunofluorescence, sections were incubated with secondary goat anti-mouse/Alexa 488 antibody (diluted 1 : 200, Invitrogen, Karlsruhe, Germany) and DAPI (diluted 1 : 1000, Sigma). Afterwards, sections were incubated for 1 h with anti- α smooth muscle actin-Cy3 (diluted 1 : 100, Sigma). For immunoperoxidase staining sections were incubated with horseradish peroxidase-labelled ready-to-use goat anti-mouse immunoglobulin antibody (diluted 1 : 200, Dako), developed with AEC substrate (Dako) and weakly counterstained with haematoxylin.

Matrigel assay

Matrigel® (Becton Dickinson, Heidelberg, Germany) was thawed on ice overnight and spread evenly over each well (30 µl) of a 24-well plate. Plates were incubated for 30 min at 37 °C to allow the Matrigel to solidify. Thirty thousand HUVECs or EPCs were seeded in triplicate into each well in 500 µl of ECGM (Promocell) supplemented with 10% FCS and supplement mix. Plates were then incubated at 37 °C and 5% CO₂ in a humidified atmosphere for 17 h. Then, cells were fixed in 4% formalin. Images were captured at $\times 100$ magnification and analysed using freeware image analysis program Image J (<http://rsb.info.nih.gov>). For quantification, representative fields from each group ($n = 3$) were taken and total length of resultant tube per field was calculated, as well as number of branch points. Results for different experimental groups were expressed as mean \pm standard deviation from three independent experiments.

Generation of endothelial spheroids

HUVEC and EPC spheroids (500 cells/spheroid) were generated as previously described (16). Cells were suspended in culture medium containing 0.25% (w/v) methylcellulose and were seeded on plastic dishes as hanging drops to allow overnight spheroid aggregation. Under these conditions,

all suspended cells contributed to formation of a single spheroid per drop, of defined size and cell number.

Implantation of spheroids into SCID mice

Four- to 6-week-old SCID mice (C.B.-17-SCID, Harlan Winkelmann, Borchon, Germany) served as recipients of cell-seeded matrices. German regulations for care and use of laboratory animals were observed at all times. All experiments were approved by the animal care committee of the University of Freiburg. Animals were housed in the veterinary care facility of the University of Freiburg Medical Centre. Implantation of spheroids was performed as previously described (7). In brief, spheroids were harvested, washed in endothelial cell basal medium (Promocell), centrifuged and mixed in 500 µl Matrigel (growth factor reduced; BD Biosciences, Heidelberg, Germany) and fibrinogen (final concentration 2 mg/ml; Merck, Darmstadt, Germany) containing angiogenic growth factors VEGF and bFGF (500 ng each; R&D Systems, Wiesbaden, Germany). Thrombin (0.4 U, Merck) was added to the mixture before subcutaneous injection on each side, lateral to the abdominal midline of SCID mice. One thousand spheroids were used per implant. EPC and HUVEC spheroids were either implanted individually in different animals or in the same animal on opposite sides lateral to the abdominal midline. In total, nine mice were used for the implantation studies. Twenty-one days after implantation, mice were killed and implants were retrieved.

Quantification of blood vessels

Each explant was entirely sectioned and three samples were taken from the front, the middle and the back of the scaffold. The selected sections were stained for human CD31 (Dako). Microscopic pictures were taken at $\times 100$ magnification of three randomized areas per section. CD31-positive structures were manually counted in the matrix area and calculated as vessel number per square millimetre.

Results

Immunological and functional characterization of EPCs

Human EPCs were isolated from the mononuclear cell fraction of peripheral blood samples and cultured on collagen type I-coated culture dishes. After 2–4 weeks, so-called late EPC-, also referred to by other authors as outgrowth endothelial cell colonies (17), were visible with typical cobblestone morphology (Fig. 1a). Cells were expanded from few in number to a monolayer, and showed multiple population doublings without senescence.

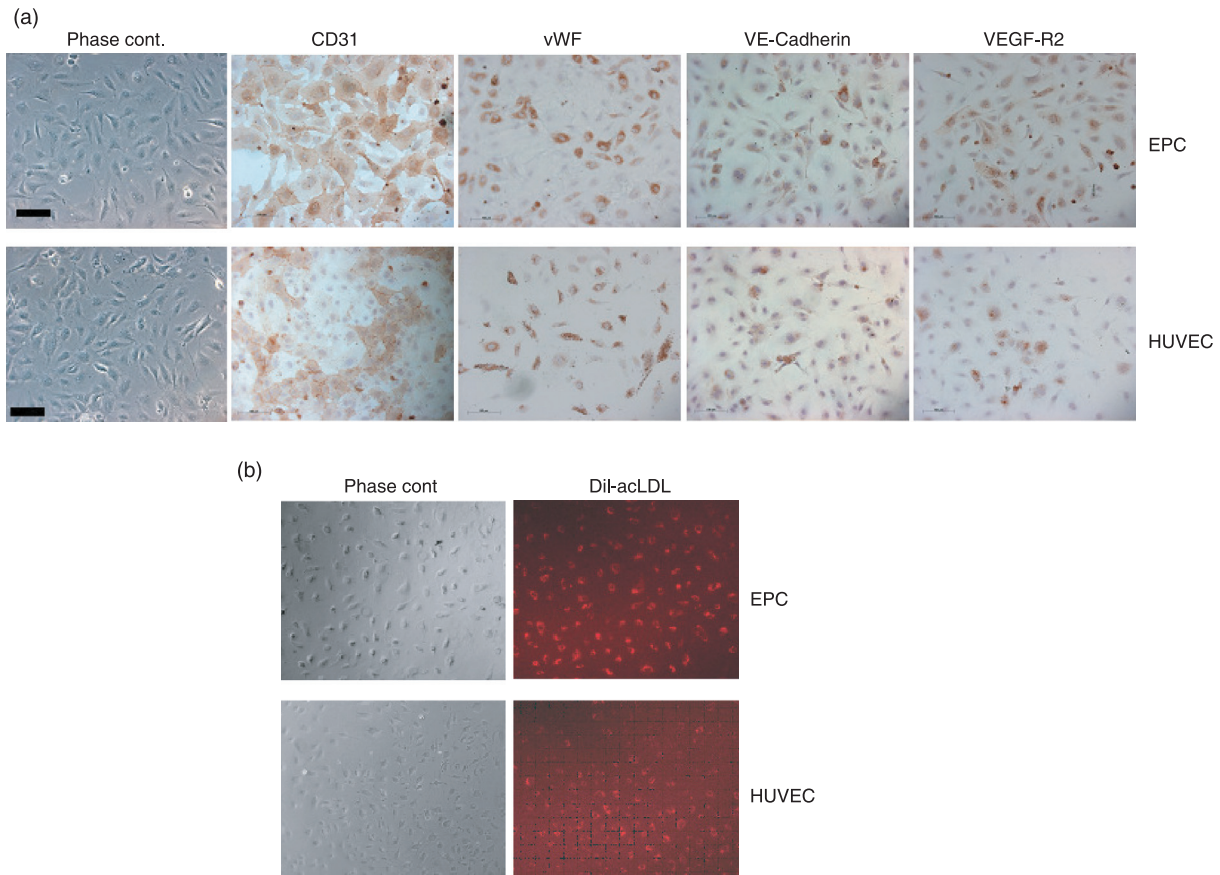


Figure 1. Morphology and immunohistochemical characterization of endothelial progenitor cells (EPC) in comparison to human umbilical vein endothelial cells (HUVEC). (a) Phase contrast images and immunohistochemical analysis of late EPCs and HUVECs for CD31, von Willebrand factor, VE-cadherin and VEGF-R2 (magnification $\times 200$, scale bar $100\ \mu\text{m}$). (b) Uptake of DiI-acLDL (red fluorescence) by late EPCs and HUVECs (magnification $\times 100$).

Endothelial phenotype of expanded EPCs was confirmed by immunohistochemistry, demonstrating expression of endothelial markers CD31, von Willebrand factor, VE-cadherin and VEGF-R2 (Fig. 1a), whereas expression of haematopoietic-specific surface antigen CD45 and monocyte/macrophage cell surface antigen CD14 were not detectable (data not shown). These results are in agreement with previous studies where EPCs have been analysed for these endothelial markers, by means of flow cytometry, reverse transcription–polymerase chain reaction and immunofluorescence (13). Moreover, expanded EPCs were also able to incorporate acLDL, which is also a hallmark of endothelial lineage cells (Fig. 1b). Of importance, EPCs were indistinguishable from HUVECs in terms of cell morphology, endothelial marker expression or acLDL uptake, demonstrating that cells isolated and expanded from adult peripheral blood were, indeed endothelial cells (Fig. 1). In addition to phenotypical characterization, we tested *in vitro* angiogenic potential of EPCs, in direct

comparison to HUVECs, in the Matrigel sprouting assay (Fig. 2). Tube formation was quantified by measuring two parameters: total tube lengths (Fig. 2a) and number of branch points (Fig. 2b). No significant difference could be detected between EPCs and HUVECs in their ability to form capillary-like sprouts *in vitro*.

In vivo vasculogenic potential of EPCs and HUVECs implanted subcutaneously into SCID mice

We have previously shown that upon implantation, HUVEC spheroids are able to form a network of stable and perfused blood vessels in immunodeficient mice without the need of co-seeding supporting mural cells (7).

In order to investigate whether EPCs would be able to form functional blood vessels *in vivo*, we implanted EPC spheroids in a Matrigel/fibrin matrix, containing angiogenic growth factors VEGF and bFGF, on one side lateral to the dorsal midline region of SCID mice. On the opposite side,

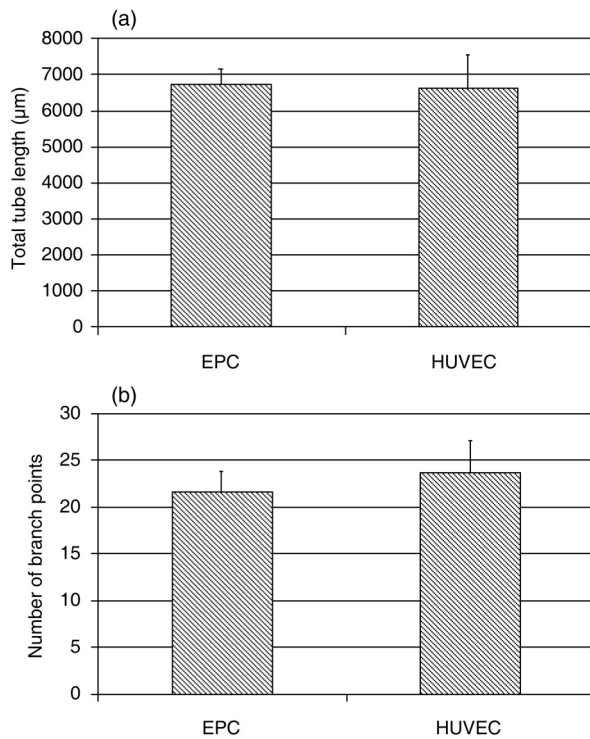


Figure 2. Quantification of *in vitro* tube formation by endothelial progenitor cells (EPC) and human umbilical vein endothelial cells (HUVEC) in Matrigel assay. Total tube length (a) and number of branch points (b) of EPCs and HUVECs grown on Matrigel for 17 h. Images used for quantification were captured at $\times 100$ magnification. Mean values \pm standard deviation from three experiments are shown.

HUVEC spheroids were implanted. This experimental design allows direct comparison of vasculogenic potential of EPCs and HUVECs in the same animal. Implants were dissected after 3 weeks and analysed for formation of human endothelial cell-derived vasculature. H&E staining revealed presence of vascular structures in high density in HUVEC implants, whereas very few microvessels were detectable in EPC implants (Fig. 3a).

Sections of implants were also immunohistochemically stained using human-specific anti-CD31 antibody, to visualize transplanted endothelial cells. This staining revealed dense networks of human CD31-positive neovessels in HUVEC implants, whereas upon implantation of EPC spheroids, very few CD31-positive microvessels could be detected (Fig. 3a). Similar results were obtained with EPC preparations from four different donors. Quantification of hCD31-positive vessel structures revealed vascular densities of 101.5 ± 39.5 microvessels/ mm^2 for HUVEC implants and 15.9 ± 3.2 microvessels/ mm^2 in EPC spheroids (Fig. 3b). Of importance, vessel densities were significantly lower in EPC implants in comparison to HUVEC implants. The immunohistochemical experiments, carried out with human-

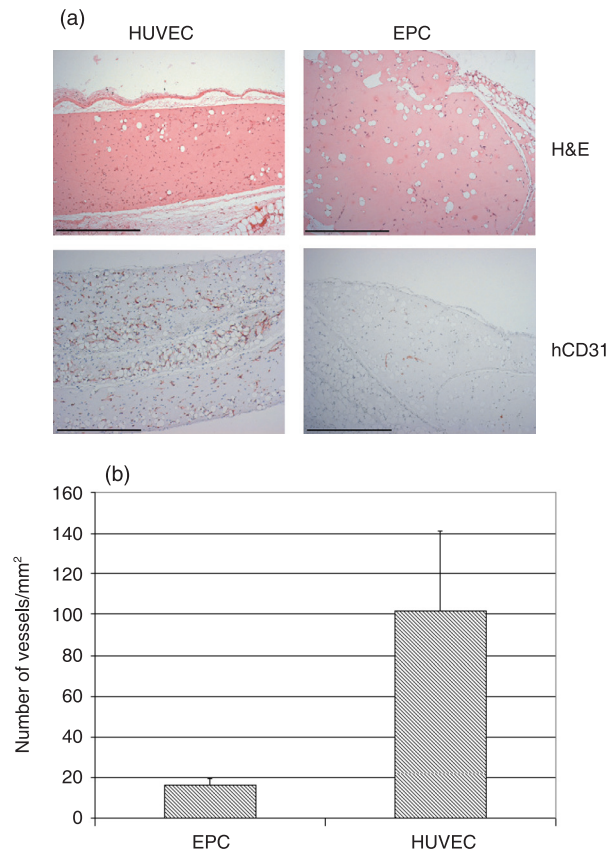


Figure 3. *In vivo* vasculogenic potential of endothelial progenitor cell (EPC) spheroids in direct comparison to human umbilical vein endothelial cell (HUVEC) spheroids. Matrigel/fibrin constructs containing EPC or HUVEC spheroids were implanted subcutaneously into SCID mice and retrieved after 3 weeks. (a) Haematoxylin and eosin (H&E) and hCD31 staining of cross-sections (magnification $\times 100$, scale bar 500 μm). (b) Microvessel density in implants was quantified by counting hCD31-positive vessel structures. Values are expressed as number of vessels per square millimetre. Means \pm standard deviations from three different constructs per experimental group are shown.

specific anti-CD31 antibody, clearly revealed that newly formed vasculature in the implants was of human origin and not formed by angiogenic ingrowth of mouse blood vessels, from surrounding mouse tissue. Human vasculature that emerged from implanted HUVEC spheroids formed anastomoses with mouse vasculature and perfused blood vessels, as evidenced by presence of intraluminal mouse erythrocytes, could be detected at higher magnification (Fig. 4). In contrast, no intraluminal mouse erythrocytes were detectable in EPC-derived vessels.

We also performed double staining for human CD34 and α -smooth muscle actin (α SMA) on sections of implants seeded with HUVEC or EPC spheroids, in order to investigate maturation of newly formed vessels. As shown in Fig. 4, newly formed HUVEC-derived blood vessels were covered

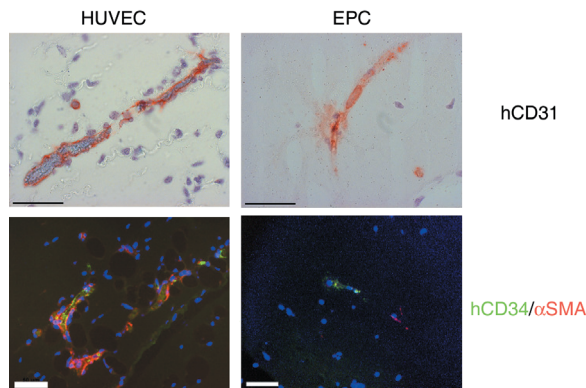


Figure 4. *In vivo* analysis of vessel maturation and perfusion. Sections from human umbilical vein endothelial cell (HUVEC) and endothelial progenitor cell (EPC) implants were stained for hCD31 and analysed for the presence of mouse red blood cells, at high magnification (magnification $\times 630$, scale bar $50\ \mu\text{m}$). Coverage of human blood vessels by mouse perivascular cells was analysed by immunofluorescence staining with antibodies against hCD34 (human-specific, green) and α -smooth muscle actin (α SMA, red). Nuclei stained with DAPI (blue) (magnification $\times 400$, scale bar $50\ \mu\text{m}$). Note α -smooth muscle actin-positive mouse cells (red) recruited to the HUVEC-derived blood vessels (green) but not to EPC-derived vessels.

with α -smooth muscle actin-positive cells, indicating that vessels were stabilized by murine pericytes or smooth muscle cells, recruited from surrounding mouse tissue. In contrast, no covering of EPC-derived neovessels by mouse perivascular cells could be detected.

In summary, our experiments showed that although EPCs and HUVECs display similar phenotypical and functional characteristics *in vitro*, they clearly differ in *in vivo* vasculogenic potential, with EPCs displaying dramatically lower ability to form stable and perfused vascular networks. These results suggest that vascular-derived endothelial cells, such as HUVECs, are superior to EPCs in terms of promoting *in vivo* vascularization of engineered tissues.

Discussion

For applications in tissue engineering, construction of stable and perfusable blood vessels is crucial for graft survival after transplantation. Without generation of a functional vascular network, implanted cells undergo cell death due to limitation in oxygen and nutrient supply. It is known from the literature that tissue vascularization can be promoted by cell-based approaches using vascular-derived endothelial cells (5,6,18). HUVECs seeded into collagen/fibronectin gels have been shown to be able to form functional blood vessels after implantation into immunodeficient mice (5). However, this process was only effective when cells were transduced with caspase-resistant

Bcl-2, which leads to delay in apoptosis and to recruitment of perivascular α -smooth muscle actin-expressing mouse cells, and hence, to stabilization of the newly formed vessels. Recent work from other groups suggests that stable blood vessels can only be formed *in vivo* when HUVECs are co-implanted with other cell types having potential to stabilize the neovessels. This has been shown in co-implantation studies of HUVECs with mesenchymal precursor cells (6) or embryonic fibroblasts (18). In both cases, co-implanted cells differentiated *in vivo* into supporting mural cells and contributed to maturation and stabilization of the engineered blood vessels. These results suggest that durable blood vessels, in a tissue engineering setting, can only be formed when implanted endothelial cells undergo the complete angiogenic cascade, consisting of vessel assembly, maturation and stabilization (19–21). We have recently reported an implantation technique based on HUVECs grown in three-dimensional spheroidal configuration (7); this model was originally developed as an *in vitro* assay for studying endothelial cell differentiation and maturation (16,22). The finding that spheroid aggregation stabilized endothelial cells and rendered them responsive to survival factors, led to the assumption that endothelial cell spheroids could be used as focal starting points for sprouting of capillaries *in vivo*. Indeed, upon subcutaneous implantation of HUVEC spheroids immobilized in a Matrigel/fibrin matrix supplemented with angiogenic growth factors VEGF and bFGF into SCID mice, development of a complex network of perfused human neovessels could be observed (7). Of importance, *in vivo* vascularization emerging from implanted HUVEC spheroids was evident in the absence of co-implanted supporting perivascular cells.

We therefore intended to investigate whether EPCs isolated from peripheral blood and implanted in spheroidal conformation would have similar vasculogenic *in vivo* potential as HUVECs. In this context, we used the HUVECs as a kind of positive control in our xenotransplantation assay. However, whereas implanted HUVEC spheroids gave rise to formation of stable and dense networks of perfused microvessels, implanted EPC spheroids showed a significantly lower ability to form vascular sprouts *in vivo*. Moreover, the few vascular structures emerging from implanted EPC spheroids were neither perfused with erythrocytes, nor covered by mouse mural cells.

Absence of perivascular mural cell coverage of EPC-derived neovessels was of particular interest. It is well established that endothelial cells produce factors, such as PDGF-BB (23,24), hepatocyte growth factor (25) and transforming growth factor-beta (19), which play important roles in recruitment of pericytes or smooth muscle cells during vascular maturation. It may be interesting in future experiments to investigate whether absence of mural cell

coverage of implanted EPCs may be related to reduced expression of these chemoattractant molecules.

Our result on the impaired ability of implanted EPCs to form functional vessels is in agreement with recent reports from other groups, where peripheral blood- or cord blood-derived EPCs were tested for their *in vivo* vasculogenic potential. Au *et al.* (26) implanted EPCs in collagen/fibronectin gel onto the pial surface in cranial windows of SCID mice and saw that implantation of EPCs alone was not effective in forming stable vasculature. Similarly, cord-derived EPCs dispersed in Matrigel matrix and implanted subcutaneously into immunodeficient mice, failed to develop microvessels (27). However, the same authors have shown that upon co-implantation of 10T1/2 cells (26) or human saphenous vein smooth muscle cells (27) EPCs were able to develop stable vascular networks *in vivo*. These results indicate that, as previously demonstrated for HUVECs implanted in single cell configuration, *in vivo* formation of durable vessels from implanted EPCs is strongly dependent on co-seeding of supporting perivascular cells.

In our study, we observed impaired vasculogenic potential of EPCs after 3 weeks of *in vivo* growth. This raises the question of whether the lower ability of EPCs to form vascular structures is the result of impaired vessel assembly or of enhanced disintegration of neovessels. Results from Au *et al.* strongly support the hypothesis that low vessel density in EPC implants is the consequence of reduced vessel-forming potential of EPCs (26).

The observed difference in *in vivo* vasculogenic potential between spheroidal HUVECs and EPCs reported here emphasizes an important difference of *in vivo* function between vascular-derived endothelial cells and EPCs. However, it should be noted that our experiments were performed in a xenogenic transplantation model and cannot be completely transferred to an autologous setting. Therefore, it is difficult to predict usefulness of this approach for direct human application. This also raises questions concerning physiological function of EPCs in neovascularization of ischaemic tissues. It is still a matter of debate to what extent EPCs contribute to new vessel formation in the adult. Several studies have shown a significant contribution of bone marrow-derived EPC to human tumour endothelium (9,28), while others were not able to define a critical role of EPCs in tumour neovascularization (29,30). Therefore in the human setting, integration of EPCs into neovessels of ischaemic tissues has not been convincingly shown. On the other hand, EPCs clearly show therapeutic potential to improve angiogenesis in patients with cardiovascular disease (31). This effect might be attributed to secretion of high amounts of angiogenic growth factors by transplanted EPCs, thereby promoting angiogenesis in previously ischaemic tissues (32,33).

In summary, our experiments support the hypothesis that EPCs are not very effective in forming stable vasculature *in vivo*. Although EPCs and vascular-derived endothelial cells, represented by HUVECs, display similar phenotypical and functional characteristics *in vitro*, EPCs clearly show less ability to form vascular networks *in vivo* upon implantation into immunodeficient mice.

Acknowledgements

We thank Beate vom Hoevel for excellent technical assistance and Arash Momeni for critical reading of the manuscript. This work was supported by funding through the Deutsche Forschungsgemeinschaft (STA-472/1-1).

References

- Shieh SJ, Vacanti JP (2005) State-of-the-art tissue engineering: from tissue engineering to organ building. *Surgery* **137**, 1–7.
- Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K *et al.* (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* **97**, 1114–1123.
- Schumacher B, Pecher P, von Specht BU, Stegmann T (1998) Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation* **97**, 645–650.
- Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, Giordano FJ, Simons M *et al.* (2001) Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease. *Am. Heart J.* **142**, 872–880.
- Schechner JS, Nath AK, Zheng L, Kluger MS, Hughes CC, Sierra-Honigmann MR *et al.* (2000) *In vivo* formation of complex microvessels lined by human endothelial cells in an immunodeficient mouse. *Proc. Natl. Acad. Sci. USA* **97**, 9191–9196.
- Koike N, Fukumura D, Gralla O, Au P, Schechner JS, Jain RK (2004) Tissue engineering: creation of long-lasting blood vessels. *Nature* **428**, 138–139.
- Alajati A, Laib AM, Weber H, Boos AM, Bartol A, Ikenberg K *et al.* (2008) Spheroid-based engineering of a human vasculature in mice. *Nat. Methods* **5**, 439–445.
- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T *et al.* (1997) Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964–967.
- Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M *et al.* (2001) Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J. Exp. Med.* **193**, 1005–1014.
- Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C *et al.* (2003) Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood* **102**, 1340–1346.
- Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B *et al.* (2001) Bone marrow cells regenerate infarcted myocardium. *Nature* **410**, 701–705.
- Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H *et al.* (2001) Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation* **103**, 634–637.
- Fuchs S, Hermanns MI, Kirkpatrick CJ (2006) Retention of a differentiated endothelial phenotype by outgrowth endothelial cells

- isolated from human peripheral blood and expanded in long-term cultures. *Cell Tissue Res.* **326**, 79–92.
- 14 Finkenzeller G, Torio-Padron N, Momeni A, Mehlhorn AT, Stark GB (2007) *In vitro* angiogenesis properties of endothelial progenitor cells: a promising tool for vascularization of ex vivo engineered tissues. *Tissue Eng.* **13**, 1413–1420.
 - 15 Siemeister G, Schirner M, Reusch P, Barleon B, Marme D, Martiny-Baron G (1998) An antagonistic vascular endothelial growth factor (VEGF) variant inhibits VEGF-stimulated receptor autophosphorylation and proliferation of human endothelial cells. *Proc. Natl. Acad. Sci. USA* **95**, 4625–4629.
 - 16 Korff T, Augustin HG (1998) Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J. Cell Biol.* **143**, 1341–1352.
 - 17 Sieminski AL, Hebbel RP, Gooch KJ (2005) Improved microvascular network *in vitro* by human blood outgrowth endothelial cells relative to vessel-derived endothelial cells. *Tissue Eng.* **11**, 1332–1345.
 - 18 Levenberg S, Rouwkema J, Macdonald M, Garfein ES, Kohane DS, Darland DC *et al.* (2005) Engineering vascularized skeletal muscle tissue. *Nat. Biotechnol.* **23**, 879–884.
 - 19 Darland DC, D'Amore PA (2001) TGF β is required for the formation of capillary-like structures in three-dimensional cocultures of 10T1/2 and endothelial cells. *Angiogenesis* **4**, 11–20.
 - 20 Carmeliet P (2003) Angiogenesis in health and disease. *Nat. Med.* **9**, 653–660.
 - 21 Jain RK (2003) Molecular regulation of vessel maturation. *Nat. Med.* **9**, 685–693.
 - 22 Korff T, Augustin HG (1999) Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J. Cell Sci.* **112**, 3249–3258.
 - 23 Abramsson A, Lindblom P, Betsholtz C (2003) Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *J. Clin. Invest.* **112**, 1142–1151.
 - 24 Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C (1999) Role of PDGF-B and PDGFR- β in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development* **126**, 3047–3055.
 - 25 Kobayashi H, DeBusk LM, Babichev YO, Dumont DJ, Lin PC (2006) Hepatocyte growth factor mediates angiopoietin-induced smooth muscle cell recruitment. *Blood* **108**, 1260–1266.
 - 26 Au P, Daheron LM, Duda DG, Cohen KS, Tyrrell JA, Lanning RM *et al.* (2008) Differential *in vivo* potential of endothelial progenitor cells from human umbilical cord blood and adult peripheral blood to form functional long-lasting vessels. *Blood* **111**, 1302–1305.
 - 27 Melero-Martin JM, Khan ZA, Picard A, Wu X, Paruchuri S, Bischoff J (2007) *In vivo* vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood* **109**, 4761–4768.
 - 28 Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L *et al.* (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat. Methods* **7**, 1194–1201.
 - 29 Göthert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM *et al.* (2004) Genetically tagging endothelial cells *in vivo*: bone marrow-derived cells do not contribute to tumor endothelium. *Blood* **104**, 1769–1777.
 - 30 De Palma M, Venneri MA, Roca C, Naldini L (2003) Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nat. Med.* **9**, 789–795.
 - 31 Assmus B, Schächinger V, Teupe C, Britten M, Lehmann R, Döbert N *et al.* (2002) Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* **106**, 3009–3017.
 - 32 Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK *et al.* (2004) Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler. Thromb. Vasc. Biol.* **24**, 288–293.
 - 33 Rehman J, Li J, Orschell CM, March KL (2003) Peripheral blood 'endothelial progenitor cells' are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* **107**, 1164–1169.