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# Vascular wall resident progenitor cells: a source for postnatal vasculogenesis

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Here, we report the existence of endothelial precursor (EPC) and stem cells in a distinct zone of the vascular wall that are capable to differentiate into mature endothelial cells, hematopoietic and local immune cells, such as macrophages. This zone has been identified to be localized between smooth muscle and adventitial layer of human adult vascular wall. It predominantly contains CD34-positive (+) but CD31-negative (-) cells, which also express VEGFR2 and TIE2. Only few cells in this zone of the vascular wall are positive for CD45. In a ring assay using the fragments of human internal thoracic artery (HITA), we show here that the CD34<sup>+</sup> cells of the HITA-wall form capillary sprouts *ex vivo* and are apparently recruited for capillary formation by tumor cells. New vessels formed by these vascular wall resident EPCs express markers for angiogenically activated endothelial cells, such as CEACAM1, and also for mature endothelial cells, such as VE-cadherin or occludin. Vascular wall areas containing EPCs are found in large and middle sized arteries and veins of all organs studied here. These data suggest the existence of a 'vasculogenic zone' in the wall of adult human blood vessels, which may serve as a source for progenitor cells for postnatal vasculogenesis, contributing to tumor vascularization and local immune response.

**KEY WORDS:** Rat, Vasculogenesis, VEGFR

## INTRODUCTION

Angiogenesis is defined as outgrowth of new vessels from pre-existing blood vessels (Carmeliet, 2003; Folkman, 2003; Jain, 2002). Main steps of this process comprise endothelial cell migration, proliferation and tube formation. Accumulating data indicate a role for circulating (C-EPC) and/or bone marrow-derived endothelial precursor cells (BM-EPC) involved in the new blood vessel formation, (Asahara et al., 1997; Asahara and Isner, 2002; Gehling et al., 2000; Grant et al., 2002; Pelosi et al., 2002), a process defined as postnatal vasculogenesis. Although homing of C-EPCs at sites of new vessel formation has been shown, up to now the exact role that BM-EPCs play in the tissue neovascularization has been a matter of debate (Asahara et al., 1999; Bagley et al., 2003; Carmeliet and Luttun, 2001; Rajantie et al., 2004). Recent data demonstrate that BM-EPC are recruited to the vessel wall rather than to endothelium (Rajantie et al., 2004). More recent data suggest a role for tissue-bound precursor cells as source of vascular cells and concomitantly accumulating macrophages during collateral vessel growth (Khmelevski et al., 2004). The existence of local tissue-bound adult precursor cells has been reported for different organs (Aarum et al., 2003; Conboy et al., 2003) but not yet for the wall of adult blood vessels (Asahara and Kawamoto, 2004). Our data suggest the existence of a 'vasculogenic zone' in the adult human vascular wall that is, to our knowledge, the first reported location for EPCs outside the bone marrow.

## MATERIALS AND METHODS

### Growth factors and antibodies

Antibodies against CD34, CD31, CD105, CD45, CD68, von Willebrand Factor, VEGFR2, TIE2, VE-cadherin and occludin were purchased from DAKO (Glostrup, Denmark) and Santa Cruz Biotechnology (CA, USA). VEGF (vascular endothelial growth factor) and FGF2 (fibroblast growth factor 2) were purchased from R&D systems (R&D systems, Wiesbaden, Germany). The monoclonal antibody against CD133 was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

### Immunohistochemical analysis

Immunohistochemical staining for CD34, CD31, CD45 (final dilution: 1:50), CD105, CD133, occludin (final dilution 1:25), CD68 (final dilution 1:250), von Willebrand Factor, TIE2 (final dilution 1:200), VEGFR2, VE-cadherin (final dilution 1:100),  $\alpha$ -smooth-muscle-actin (final dilution 1:400) and CEACAM1 using the antibody 4D1/C2 (final dilution: 1:400) was performed on paraffin wax sections obtained from 10 different human internal thoracic arteries before and after the ring assay using antibodies recognizing endothelial and precursor cell markers mentioned above. After de-paraffinization and rehydration, the sections were subjected to immunohistochemistry. The nickel-enhanced glucose oxidase technique was used to develop the immunostaining as previously described (Davidoff and Schulze, 1990; Kilic and Ergun, 2001). Negative controls were performed by omitting or replacing the primary antibodies with Ig-matched isotypes or PBS. All sections were counterstained with Calcium Red for 1-2 minutes to visualize the tissue structure.

In two cases, cells that sprouted into the collagen gel from HITA-wall during ring assay were isolated from the collagen gel, subsequently cultured in endothelial growth medium (MV Medium, PromoCell, Heidelberg, Germany) flasks and then transferred into chamber slides and used for immunocytochemistry to detect VEGF receptors, the angiopoietin receptor TIE2 and CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1). On some sections, double immunostaining was performed for VEGFR2 and CEACAM1 combining the glucose oxidase technique as described above and immune fluorescence method using the TRITC-conjugated secondary antibody.

Apoptotic rat macrophages were double stained with a PE-labeled anti-ED2 (Serotec) antibody and an anti-caspase 3 antibody (R&D Systems). For detection of immature endothelial cells, specimens were double stained with a polyclonal rabbit anti FLK1 antibody and a polyclonal goat anti VE-

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cadherin antibody (both from Santa Cruz, CA, USA). As secondary antibodies we used a biotinylated donkey anti-goat followed by rhodamine conjugated streptavidine (both from Dianova, Hamburg, Germany) for the VE-cadherin antibody and a Cy2-conjugated goat anti rabbit antibody (Dianova) for the FLK1 antibody. Negative controls were performed by omitting the first antibody.

#### Dispersion cell culture

Dispersion cell culture preparation was performed essentially according to a technique described by Brewer (Brewer, 1997). Tissue pieces were minced and placed into PBS buffer. After centrifugation for 10 minutes at 230 g, tissues were subsequently resuspended in digestion solution containing trypsin/EDTA at 37°C for 5 minutes. Non-digested tissues were removed by filtration. The suspension was centrifuged for 5 minutes at 230 g. The cell suspension was placed into endothelial growth culture medium (MV Medium, PromoCell), washed twice with endothelial growth culture medium, resuspended in culture medium and plated on collagen- or fibronectin-coated wells using six-well culture dishes (Nunc, Wiesbaden, Germany; 2 ml of cell suspension per well). After 24 hours of incubation at 37°C and 5% CO<sub>2</sub>, medium was renewed and changed every second day.

#### Ring assay

Fragments of 10 different HITAs were cut into 1 mm thick rings and embedded between two layers of collagen gel, consisting of collagen type 1, sodium hydroxide, glutamine, sodium bicarbonate, vitrogen-gel (Invitrogen) and MEM (10×) using 48-well cluster tissue culture plates. Embedded rings were incubated at 37°C. The medium was changed every third day. Rings were evaluated every day using a phase contrast microscope (Zeiss, Jena, Germany) equipped with a digital camera (Zeiss, Jena, Germany). In three experiments, rings were fixed in Bouin's solution and embedded in paraffin wax for immunohistochemistry. In two experiments, rings were fixed in glutaraldehyde (5.5%), subsequently embedded in Epon 812 and used for electron microscopic studies.

To monitor the cells of the HITA-wall during capillary sprouting within collagen gel, we introduced 1 µl of an adenoviral construct (AdV5-GFP) containing the reporter gene GFP into the outer layer of the HITA-wall under observation with stereo microscope in two cases. Prior to this, the rings have been placed into the first layer of collagen gel that was prepared in the wells of a 48-well cluster culture dish. After addition of the second layer of collagen gel the rings were observed twice daily using an inverse fluorescence microscope (Zeiss, Jena, Germany) equipped with a digital camera (Zeiss). For the preparation of adenoviral construct containing GFP gene we used the homologous recombination technique of adenoviral vectors (He et al., 1998) as we also reported previously (Ergun et al., 2000).

#### In vitro endothelial tube assay

Cells isolated from collagen gel after performing the ring assay were cultured in endothelial growth medium (MV Medium, PromoCell) containing 5% FCS until confluence. They were then trypsinized and seeded on the pre-prepared polymerized collagen gel in 48-well cluster tissue culture dishes as described previously (Ergun et al., 2000). At different stages of confluence, the full MV medium was replaced by hunger medium containing the 2% FCS without supplements. After 24 hours of culture, the cells in a part of the wells were stimulated with VEGF (50 ng/ml) and FGF (10 ng/ml). They were then observed and photographed using an inverse microscope (Zeiss) equipped with a digital camera.

#### Flow cytometry

Cells isolated from HITA wall and ring assays were incubated with phycoerythrin (PE)-conjugated anti-CD34, anti-CD31, anti-VE-cadherin (all from Pharmingen, Hamburg, Germany) or AC133 monoclonal antibodies (Miltenyi Biotec). For two-color flow cytometry, FITC-conjugated anti-CD105 (Pharmingen) or vWF (Serotec, Düsseldorf, Germany) MoAbs were used as described (Gehling et al., 2000). Isotype-matched mouse immunoglobulin served as controls. All incubations were performed at 4°C. Cells were incubated with the MoAb for 30 minutes in the presence of normal goat serum. After each incubation, cells were washed in PBS containing 0.1% BSA. Single- and two-color flow cytometric analyses were

performed, using a FACS SCAN flow cytometer (Becton Dickinson, Heidelberg, Germany) and Cell Quest software (Becton Dickinson). Each analysis included at least 5000 events.

#### Depletion of CD105<sup>+</sup> mature endothelial cells from HITA wall

Cells isolated from HITA walls were incubated with CD105-conjugated super paramagnetic microbeads (Miltenyi Biotec), washed and processed using the VarioMacs system as previously described. Purified cells were discarded, except for an aliquot that was analyzed by flow cytometry.

#### Electron microscopy

The arteries used in the ring assay were perfused with 5.5% phosphate-buffered glutaraldehyde under manual pressure after rinsing with 0.15 M phosphate-buffered saline. Small blocks of these arteries were excised and immersed for 8 hours in the same fixative, followed by fixation in 1% OsO<sub>4</sub> for 2 hours. The blocks were then embedded in Epon 812. Serial semithin sections (1 µm) were stained with Toluidine Blue/Pyronin. For electron microscopy, ultrathin sections (about 80 nm thick) of perfused arteries were obtained using a Porter MTB2 ultramicrotome contrasted with uranyl acetate and lead citrate, and examined using a Philips EM 300 (Eindhoven, Netherlands) transmission electron microscope.

#### In vivo studies

The right femoral artery was occluded in Sprague Dawley rats (400-500 g) as previously described (Ito et al., 1997). Bone marrow depletion was induced with intraperitoneal injections of cyclophosphamide (80 mg/kg bodyweight 5 days prior to occlusion followed by an additional injection of 40 mg/kg bodyweight 3 days prior to occlusion), as previously described (Khmelewski et al., 2004).

#### Isolation and cultivation of tissue resident macrophages

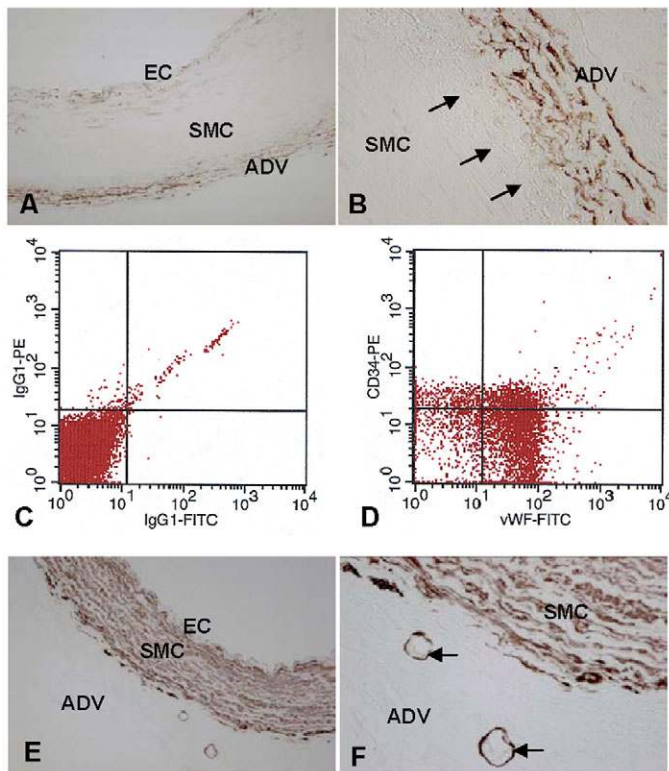
Tissue pieces of collateral arteries were minced in CBFHH buffer and subjected to numerous rounds of trypsin and DNase digestion as previously described. After filtration (30 µm filter), cells were incubated with an anti-ED2 antibody (Serotec), followed by incubation with IgG-conjugated microbeads (Miltenyi Biotec). Magnetically labeled cells were purified using a VarioMacs as previously described (Gehling et al., 2000). Cells were plated in a chamber slide and cultured in serum-free Macrophage Medium (Invitrogen, Karlsruhe, Germany).

#### Proliferation assay

Isolated ED2<sup>+</sup> cells were grown in Macrophage SMF Medium (Invitrogen) that was supplemented with macrophage-colony stimulating factor. As positive control, CD133<sup>+</sup> cells isolated from G-CSF mobilized peripheral blood were cultured and expanded as previously described (Loges et al., 2004). Immunoselected CD133<sup>+</sup> cells and ED2<sup>+</sup> cells were incubated with BrdU (0.3 mg/ml) for 3 days and were subsequently stained using the BrdU Flow Kit (Pharmingen). Positive staining was evaluated using a Zeiss fluorescence microscope (Zeiss, Jena, Germany).

## RESULTS

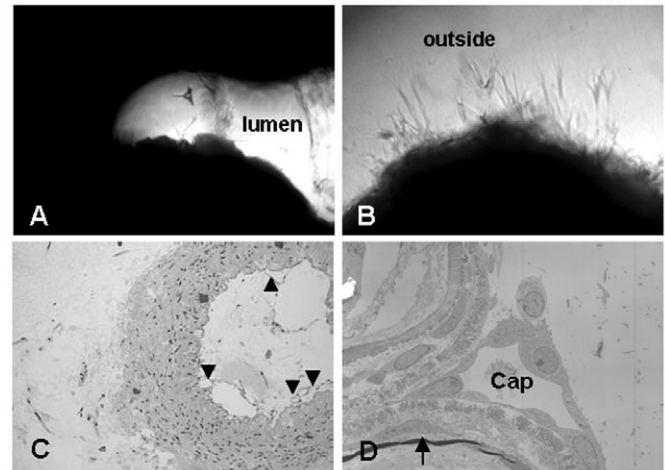
The interesting issue was whether EPCs can also exist in the wall of adult human blood vessels. The experimental approach to address this requires appropriate human material, which is in very limited supply or not easy available because of ethical principles. Human internal thoracic artery (HITA) is frequently used in coronary artery bypass grafting and fragments of it can be used in experimental studies without additional burden for the patients. In a first step, we wanted to characterize the vascular cells immunohistochemically using several markers for endothelial and smooth muscle cells. These studies revealed to our surprise the existence of CD34<sup>+</sup> CD31<sup>-</sup> cells located at the border between the smooth muscle and adventitial layer of the vascular wall (Fig. 1A,B), mostly localized in close vicinity to the external elastic membrane of the vascular wall. The existence of CD34<sup>+</sup> cells in the wall of HITA was confirmed by FACS analysis of cells obtained by trypsinization of the vascular wall and subsequent removal of



**Fig. 1. Mural CD34<sup>+</sup> cells in HITA wall.** Besides the expected positive staining for CD34 in the endothelial layer (EC), CD34<sup>+</sup> cells were found at the transition area from the smooth muscle cell (SMC) to adventitial layer (ADV) (A). Higher magnification reveals the close proximity of these cells to the external elastic membrane (arrows) of the vascular wall (B). FACS analyses on cells isolated from HITA wall after removal of mature endothelial cells positive for CD105 confirm the existence of mural CD34<sup>+</sup> cells that are partially positive for von Willebrand Factor (vWF) (C,D). (E,F) Immunostaining for  $\alpha$ -smooth muscle actin revealed a clear staining of smooth muscle cells in the HITA wall (SMC) (E) and in the wall of vasa vasorum (arrows in F) (F) of HITA. No staining is visible in the CD34<sup>+</sup> zone of HITA wall, shown at higher magnification in F.

mature endothelial cells using CD105-conjugated microbeads (Fig. 1C,D). The complete depletion of CD105<sup>+</sup> cells was confirmed by FACS analysis (not shown). Further studies showed that these cells are negative for  $\alpha$ -smooth muscle actin (Fig. 1E,F), indicating that they do not belong to the vascular smooth muscle cells. In this mural CD34<sup>+</sup> cell zone, KDR<sup>+</sup> (VEGFR2<sup>+</sup>) and TIE2<sup>+</sup> cells were also found, whereas no staining was detected for CD105 (endoglin, a TGF $\beta$  receptor) and VE-cadherin (not shown). Both CD34 and CD31 marked endothelial cells lining the lumen of HITA and the lumina of vasa vasorum as expected (not shown). Similar results were obtained after immunostaining for von Willebrand Factor (vWF) (not shown).

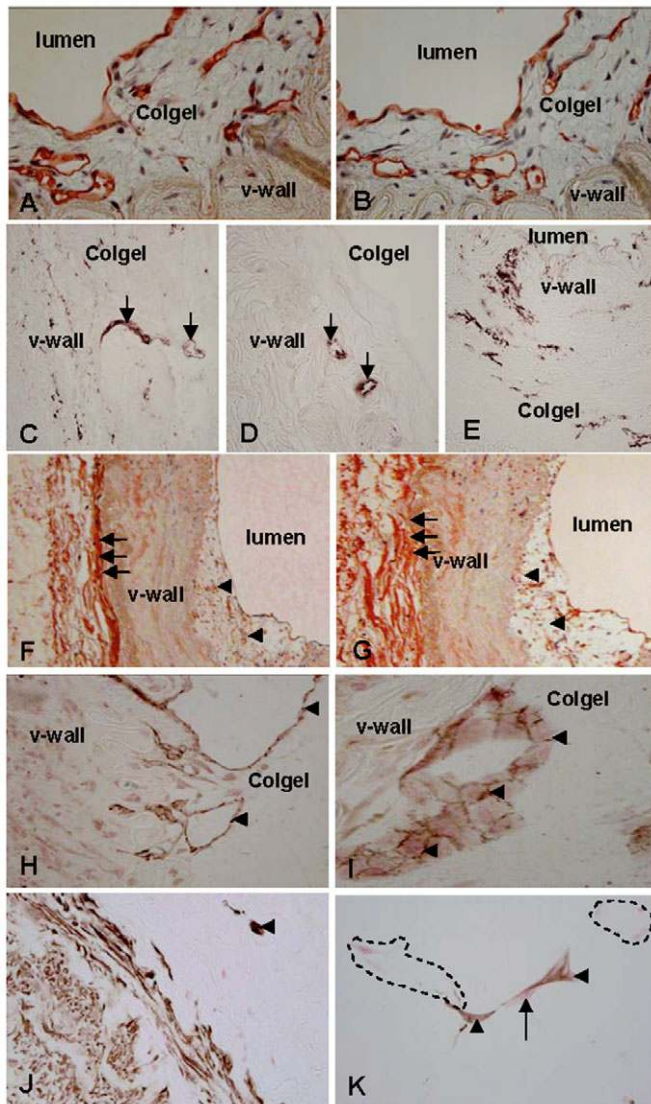
To determine whether the mural CD34<sup>+</sup> cells are involved in the outgrowth of new vessels, we performed a modified arterial ring assay using collagen type I gel or matrigel as matrix in which HITA rings of 1-2 mm thickness were embedded (Nicosia and Ottinetti, 1990). After 5-7 days of culture in the gel, we found capillary-like outgrowth into the vessel lumen as well as into the periphery of the rings (Fig. 2A,B). Histological sections confirmed the capillary formation (Fig. 2C). Finally, electron microscopic studies revealed the establishment of inter-endothelial contacts (Fig. 2D).



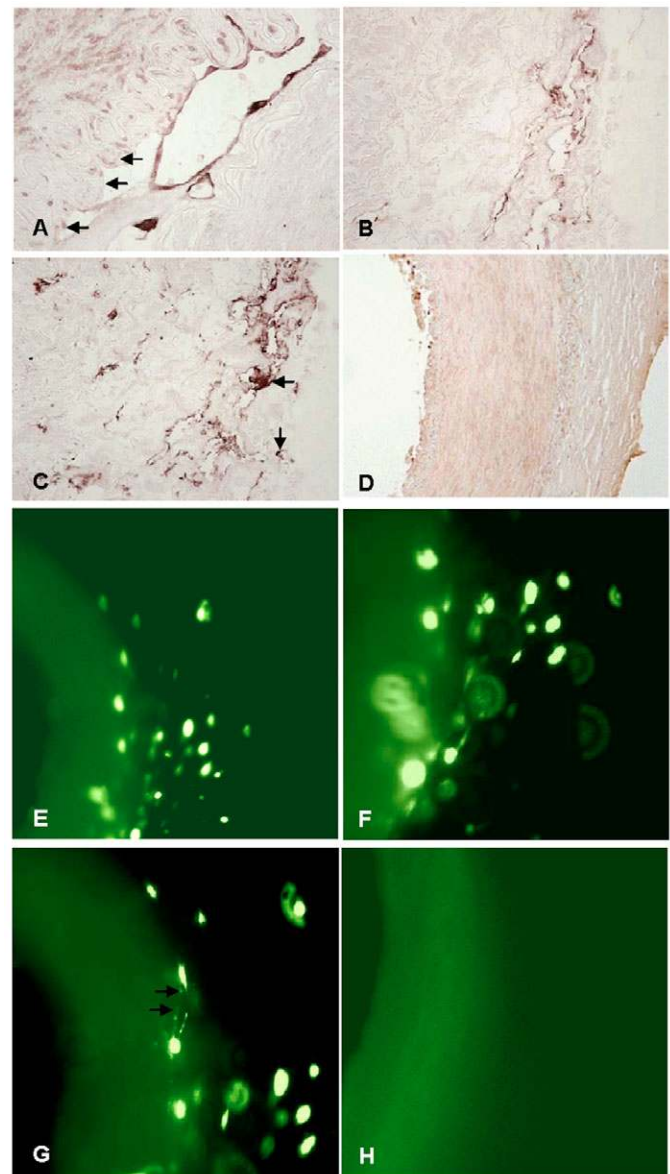
**Fig. 2. Capillary sprouts from the HITA wall.** HITA rings embedded in collagen gel or in matrigel show extensive capillary sprouting into the lumen (A) and outside the rings (B). Light microscopical studies on paraffin wax sections and electron microscopic studies on fine sections of HITA after the ring assay demonstrate the formation of tubular structures (arrowheads) in the lumen and outside the rings (C). The formation of capillaries (Cap) is also confirmed by electron microscopic analysis (D). New capillaries develop in close contact with mature endothelial cells based on the internal elastic membrane (arrow).

Next, we performed immunohistochemistry for CD34 and CD31 to determine whether the vascular wall resident CD34<sup>+</sup> cells are involved in the capillary outgrowth. Although new capillaries in the lumen of HITA rings (obviously formed by cells originating from the pre-existing endothelial cells) expressed both CD34 and CD31 (Fig. 3A,B), capillary sprouts at the outside of HITA rings were only positive for CD34 (Fig. 3C), while CD31 immunostaining was detectable only in endothelial cells lining vasa vasorum (Fig. 3D). When cultures of HITA rings were maintained for a long time, for example for more than 2 weeks, the CD34<sup>+</sup> cells formed capillary channels through the entire vascular wall (Fig. 3E). Further immunohistochemical studies on sections obtained from paraffin wax-embedded HITA fragments after ring assay demonstrated that cells forming tubes into the HITA-lumen, and into the outside of the HITA rings and cells localized in the CD34<sup>+</sup> zone of HITA wall, were positive for endothelial specific markers VEGFR2 (Fig. 3F) and TIE2 (Fig. 3G), a receptor for angiopoietin 1 and angiopoietin 2. Although no VE-cadherin<sup>+</sup> cells were found in the CD34<sup>+</sup> cell zone of HITA wall, cells involved in the capillary formation of HITA rings expressed VE-cadherin (Fig. 3H,I). In some capillaries grown in the collagen gel, occludin, a tight-junction protein, was also found (not shown). These data indicate that, similar to the reported behavior of C-EPCs and BM-EPCs, vascular wall resident CD34<sup>+</sup> endothelial precursor cells (VW-EPCs) have the capacity to differentiate into mature endothelial cells *ex vivo*. Furthermore, the immunostaining for  $\alpha$ -smooth muscle on HITA sections after ring assay demonstrated that smooth muscle cells are detectable only sporadically in the adventitial layer and within collagen gel outside the rings (Fig. 3J,K). Interestingly, within collagen gel, they were mostly tightly associated to the capillary-like tubes formed by CD34<sup>+</sup> cells.

Immunostaining for CEACAM1, a cell-cell adhesion molecule with angiogenic properties that is upregulated in endothelial cells of only angiogenically activated or newly formed capillaries, and for CD34 using serial sections of HITA after the ring assay showed the presence of CEACAM1 in new endothelial sprouts within the vessel

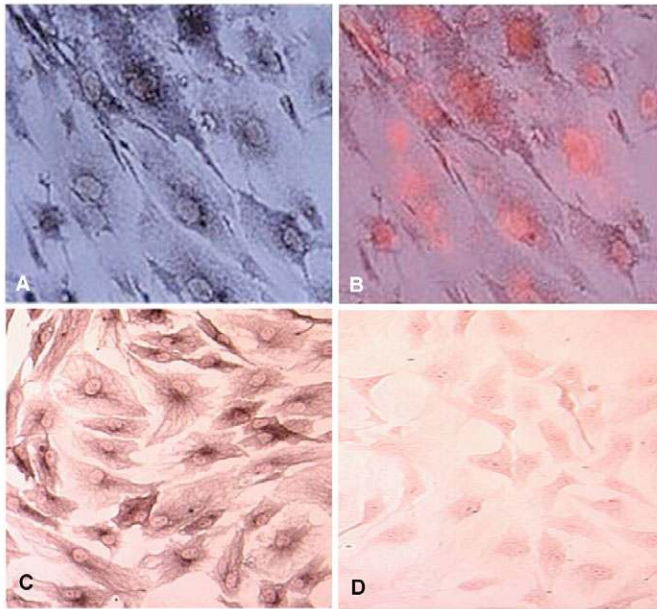


**Fig. 3. Capillary formation by and in vitro maturation of CD34<sup>+</sup> VW-EPC.** Immunostaining on sections of paraffin wax-embedded HITA rings after being subjected to ring assay demonstrates that intraluminal new capillaries exhibit both CD34 (A) and CD31 (B), whereas capillary outgrowth within the wall of and outside the HITA rings stains only positive for CD34 (arrows) (C). CD31 is detectable in vasa vasorum (arrows) (D) but not in new vessels at this stage of vascular formation. Capillarization through the entire vascular wall, as indicated by positive staining for CD34, occurs when rings were cultured for more than 14 days (E). Cells located in the CD34<sup>+</sup> vasculogenic zone (arrows) and cells forming new tubes within the HITA lumen (arrowheads) exhibit immunostaining for VEGFR2 (F) and TIE2, the receptor for Ang1 and Ang2 (G). The endothelial marker VE-cadherin is present in mature endothelial cells lining the vascular lumen and in the newly formed vessels in the lumen (arrowheads) (H). Remarkably, VE-cadherin is also visible at the cell-cell contacts (arrowheads) of new vessel sprouts outside the rings (I). Immunostaining for  $\alpha$ -smooth muscle actin after the ring assay revealed only few positive cells within the adventitial layer (arrowhead) (J) and also within the collagen gel outside the rings (arrowheads) (K) that mostly tightly associated to the endothelial tubes (indicated by broken lines) or to single cells (arrow). (C,D,E,H,I,J,K) Counterstaining with Calcium Red; (A,B,F,G) counterstaining with Hematoxylin.



**Fig. 4. CEACAM1 expression in new endothelial sprouts.**

Immunostaining for CEACAM1 on sections of embedded HITA rings demonstrate the presence of CEACAM1-positive cells within the lumen (A) and in the outside of HITA rings (B). Mature endothelial cells lining HITA lumen and not involved in the sprouting remain negative for CEACAM1 (arrows). CD34 immunostaining of a serial section following the section in B confirms the involvement of CD34<sup>+</sup> cells (arrows) in formation of these new capillaries at the border between vascular wall and the collagen gel (C). CEACAM1 is not present in the quiescent endothelial cells lining HITA lumen or in the vasculogenic zone of HITA when ring assay was not performed (D). (E-H) GFP marking of HITA-wall cells in ring assay. The injection of Adv5-GFP into a zone of the outer layer of the HITA demonstrates GFP-marked cells within and migrating out from the vascular wall (E). Higher magnification shows the sprouts from the vascular wall into the collagen gel (F). Focusing on another level demonstrates the formation of vascular like channel (arrows) at the border between the vascular wall and collagen gel corresponding to the vascular channels presented in B and C (G). No such sprouting is visible in the control ring without GFP-Adv5 (H).



**Fig. 5. Endothelial markers in cells sprouted from HITA wall during ring assay.** Similar to that presented in the Fig. 4B cells isolated from the collagen gel after performing the ring assay also show a clear staining for CEACAM1 (A). Double immunostaining of these cells for CEACAM1 (dark) and VEGF receptor 2 (KDR) (red) demonstrate the colocalization of the VEGFR2 and CEACAM1 in the majority of the cells (B). Nearly all cells are also positive for the endothelial marker TIE2 (C). No specific staining is visible in the control section (D).

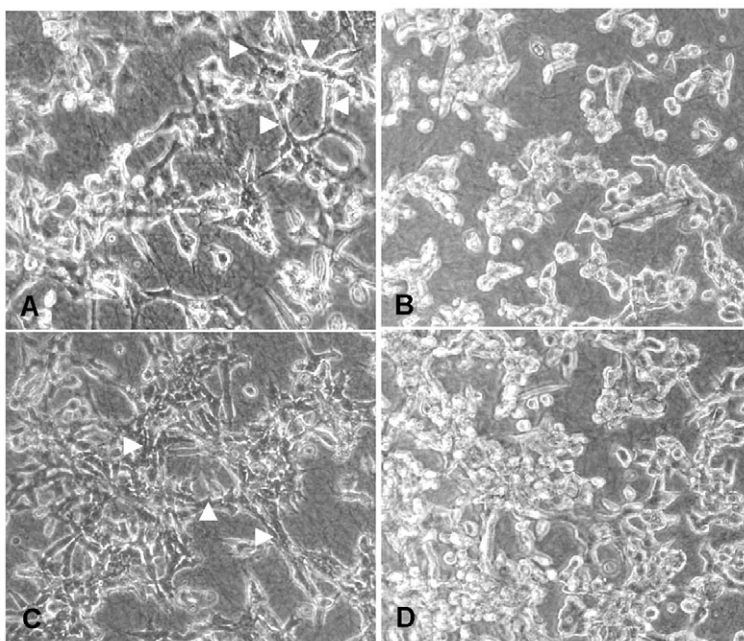
lumen (Fig. 4A) and in capillary sprouts into the outside of the rings (Fig. 4B). Immunostaining for CD34 on the serial section following the section presented in Fig. 4B confirms the formation of these capillaries by the VW-EPC (Fig. 4C). There was no CEACAM1 staining in the HITA sections without the ring assay, as expected except few granulocytes adherent to the endothelium lining vascular lumen (Fig. 4D).

To better characterize the origin and the localization of the sprouting cells from HITA-wall, we introduced 1  $\mu$ l of AdV5-GFP construct into the outer layer of the pre-prepared HITA rings. After 24 hours, we could see cells marked by green fluorescence within the outer layer and 3 days after we could also see sprouting cells into the outside of the rings (Fig. 4E). Higher magnification revealed flattened cells expressing GFP that indicate capillary-like formation (Fig. 4F). Focusing through the HITA-wall revealed capillary-like channels marked by GFP at the border between vascular wall and collagen gel (Fig. 4G). No green fluorescence marked cells were seen in the control HITA ring (Fig. 4H).

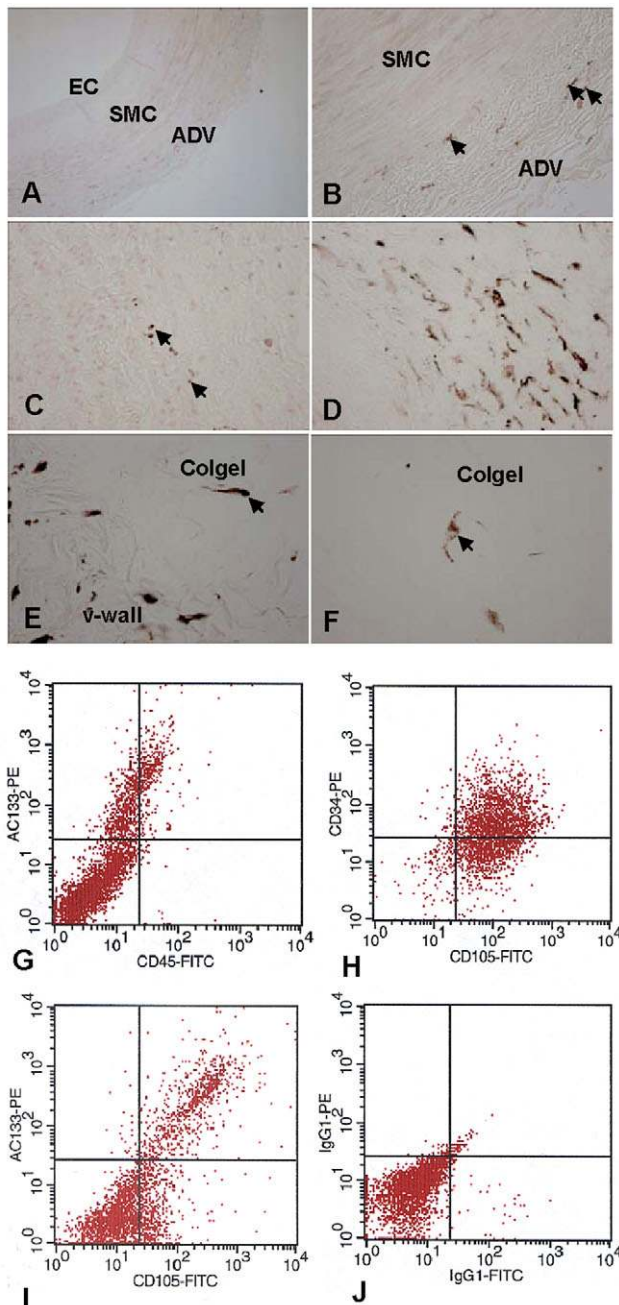
Corresponding to the immunohistochemical results above isolated cells from collagen gel after ring assay also exhibited strong staining for CEACAM1 (Fig. 5A) and the majority of them were positive for both CEACAM1 and VEGFR2 (Fig. 5B). They were also positive for TIE2 (Fig. 5C), although no specific staining was seen in the corresponding control section (Fig. 5D).

To test the capability of sprouting cells to form capillary-like tubes under in vitro conditions, the cells isolated from collagen gel after performing the ring assay were used in endothelial tube assay at different stage of cell confluence. In all cases, they formed a tight network of capillary-like tubes in response to VEGF (Fig. 6A,C) and to FGF2 (not shown), although no such tubes were observed in corresponding controls (Fig. 6B,D).

To determine whether hematopoietic and/or mesodermal cells are also present in the HITA wall, we performed immunostaining for CD14, CD133 and CD45. However, only few cells in the CD34<sup>+</sup> cell zone were also positive for the pan-leukocyte marker CD45 (Fig. 7A,B), whereas no staining was found for AC133 and CD14, probably owing to the possible failure of the antibodies in paraffin tissue. In a further step, we wanted to explore whether macrophages are present in the HITA wall and may contribute to the capillary sprouting from the HITA rings as shown above. Immunostaining for the macrophage marker CD68 on paraffin sections of untreated HITA revealed only single and randomly distributed macrophages, mostly associated with the lumina or the wall of vasa vasorum (Fig. 7C) but not located in the CD34<sup>+</sup> cell zone. Unexpectedly, the number of CD68<sup>+</sup> cells within the HITA wall dramatically increased



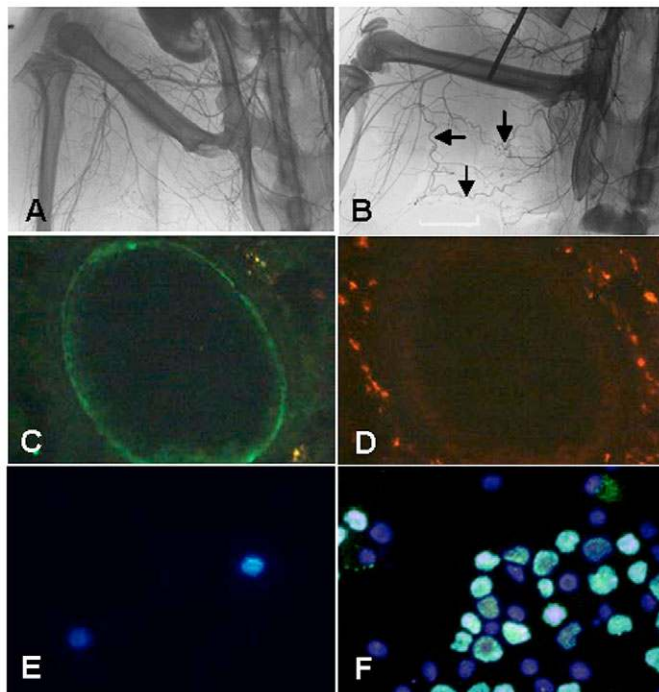
**Fig. 6. Capillary-like tube formation in vitro by cells sprouted from HITA rings.** At different stages of confluence (A,B: non confluent; C,D: subconfluent), cells isolated from collagen gel after performing a ring assay formed a network of tubes (arrowheads) when stimulated with VEGF (A,C). No tubes were visible in controls exposed to basal medium only (B,D).



**Fig. 7. CD45 and CD68 positive cells in HITA wall before and after ring assay.** Similar to CD34<sup>+</sup> cells, CD45<sup>+</sup> cells (arrows) are localized at the transitional zone between smooth muscle cell layer (SMC) and adventitial layer (ADV) (A, at higher magnification in B), but their number is significantly lower than that of CD34<sup>+</sup> cells. No specific reaction is visible in endothelial cell layer (EC). Further immunohistochemical studies demonstrate in only a few cases sporadic and randomly distributed CD68<sup>+</sup> cells in the adventitial layer (arrows) of untreated HITA (C). They are mostly localized distant from the mural zone where CD34<sup>+</sup> and CD45<sup>+</sup> cells were found. By contrast, a significant accumulation of CD68<sup>+</sup> cells is visible in the adventitial layer including the zone containing the CD34<sup>+</sup> and CD45<sup>+</sup> cells when HITA rings were subjected to the ring assay (D). Some of these macrophages migrate into the collagen gel outside the ring (arrow) (E) and are involved in the capillary sprouts in the collagen gel (arrow) (F). FACS-analyses of cells isolated from the collagen gel after ring assay demonstrate the existence of different cell populations such as CD133<sup>+</sup> cells, CD45<sup>+</sup> cells and CD105<sup>+</sup> cells (G-I) among the cells migrated into the gel and/or involved in vessels sprouting from HITA. These FACS analyses show that approximately 20% of the CD133 positive cells exhibit CD45 (G). Unexpectedly, more than 60% of CD34<sup>+</sup> cells co-express CD105 (H), although freshly isolated cells from the HITA wall did not express CD105 when prior to these analyses the pre-existing mature endothelial cells were removed via CD105 conjugated super paramagnetic microbeads. Furthermore, CD133<sup>+</sup> cells also exhibit CD105 approximately in equal intensity (I). IgG-FITC and IgG-PE were used as control (J).

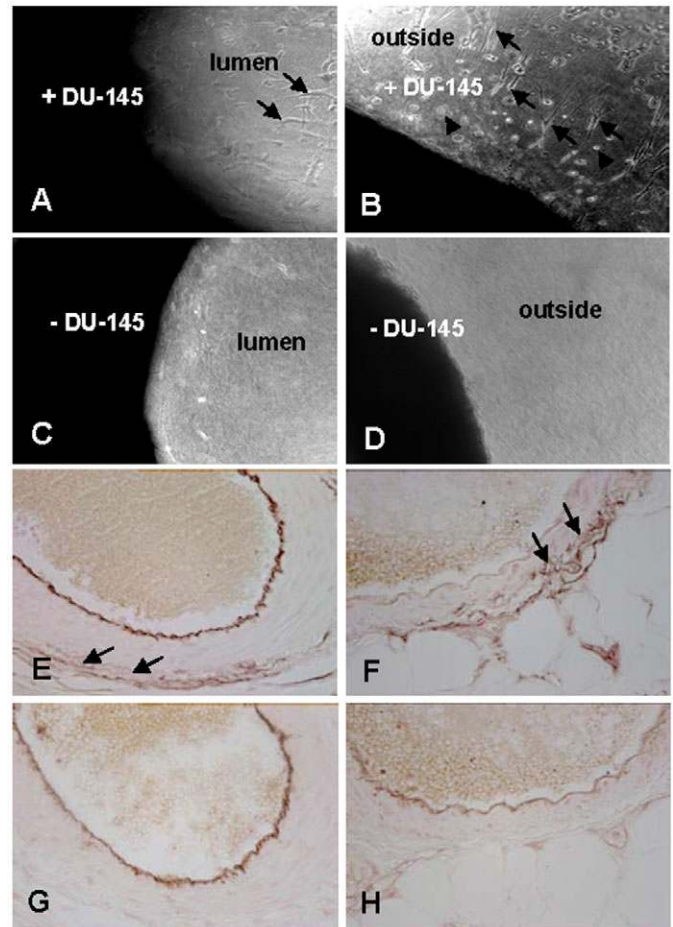
during culture of the HITA rings in collagen gel (Fig. 7D). To our surprise, this proliferation and accumulation of macrophages in the HITA rings was strongly localized in the CD34<sup>+</sup> cell zone of the HITA wall, increasing with culture time and expanded to the entire adventitial layer. Additionally, we found that macrophages migrated from this area into the gel outside the HITA rings (Fig. 7E) and few of them were involved in the capillary-like tube formation within the collagen gel (Fig. 7F). To further characterize the cells migrating into the collagen gel, we extracted them from the gel and cultured them in culture dishes to achieve a sufficient amount for FACS analysis. These studies confirmed our immunohistochemical results shown above and demonstrated the existence of CD34<sup>+</sup> cells (Fig. 7G), CD105<sup>+</sup> cells (Fig. 7H), AC133<sup>+</sup> cells (Fig. 7I) and CD45<sup>+</sup> cells (Fig. 7J). The surface expression pattern of the cells studied here is summarized in Table S1 (see supplementary material).

One of the *in vivo* models where strong accumulation of macrophages can be observed is the growth of collateral vessels after ligation of the femoral artery (Fig. 8A,B) (Khmelewski et al., 2004). Recently, it has been shown that tissue resident macrophages rather than circulating and bone marrow-derived macrophages contribute to collateral vessel growth (Khmelewski et al., 2004). Using this *in vivo* model, we obtained findings that were similar to those obtained from HITA rings studies above. Immunostaining studies on sections of collateral arteries grown in rats after femoral artery ligation and depletion of bone marrow cells by treatment with cyclophosphamide revealed an accumulation of macrophages within the wall of these arteries within 3 days after arterial occlusion (Fig. 8C). Immunostaining of these vessels for VEGFR2 marked endothelial cells lining the arterial lumen (Fig. 8D). As no considerable number of mature macrophages was found in the wall of the collateral arteries prior to their activation through femoral artery ligation and because accumulation of macrophages upon femoral artery occlusion did not differ between monocyte-competent and monocyte-deficient animals, we hypothesize that the accumulating mature macrophages in the wall of these arteries originate from vascular wall resident precursor cells. Furthermore, immunoselected tissue-resident macrophages themselves did not proliferate *in vitro* (Fig. 8E), although we were able to show that local macrophage accumulation during collateral growth occurs because of pronounced cellular proliferation. CD133<sup>+</sup> cells isolated from human peripheral blood and used as positive control show an extensive BrdU incorporation (Fig. 8F). Additional evidence for the existence of tissue-resident macrophage precursors was gained in our HITA ring assays. In the absence of circulating blood cells, there was a pronounced increase of CD68<sup>+</sup> cells, representing mature macrophages within the wall during cultivation. These cells did not only accumulate in the adventitial space but were also integrated into vascular sprouts.



**Fig. 8. In vivo accumulation of macrophages in the vascular wall after arterial occlusion.** (A,B) Post-mortem angiography shows the right femoral artery of a rat prior to (A) and 7 days after (B) its occlusion. The prominent appearance of collaterals (B, arrows) after femoral artery occlusion is seen, as reported earlier (Ito et al., 1997). (C,D) Immunofluorescence studies on sections from rat collateral arteries show KDR staining in endothelial cells (C) and the intramural accumulation of macrophages (ED2-antibody, red staining) 3 days after occlusion (D). No notable BrdU incorporation (E) is detectable in mature immunoselected macrophages from the wall of collateral arteries after femoral artery occlusion, indicating that these cells do not proliferate. CD133<sup>+</sup> cells isolated from human mobilized peripheral blood and used as positive control show an extensive BrdU incorporation (F). DAPI staining was used to visualize all cells.

BM-EPCs have been shown to contribute to tumor vascularization, indicating that tumor vessels are not only formed by angiogenesis but also by postnatal vasculogenesis (Bagley et al., 2003; Gehling et al., 2000; Ribatti et al., 2003). We wanted to explore whether the CD34<sup>+</sup> cells in the wall of HITA can be induced to capillary outgrowth by tumor cells. To reach this goal, we established a modified arterial ring assay, in which cells of the prostate cancer cell line DU-145 were implanted into a collagen gel containing the rings of HITA. Although capillary-like sprouting from untreated HITA rings was initially observable after 3-4 days of culture, the presence of DU-145 cells resulted in capillary sprouting at the outside of HITA rings, in most experiments within 24 hours of culture (Fig. 9A,B). No sprouts were seen at this time in ring cultures without tumor cells (Fig. 9C,D). The amount of capillary outgrowth at the outside of HITA rings and the very short time in which they developed suggest that they are derived from vascular wall resident CD34<sup>+</sup> EPCs rather than from sprouting of pre-existing mature endothelial cells lining the HITA lumen and/or the lumina of vasa vasorum. Our data clearly indicate that these cells can be acquired for tumor vascularization. The general relevance of VW-EPCs for tumor vascularization is that they are present not only in the wall of HITA but also in the wall of proper blood vessels of organs where primary tumors develop. Indeed, detailed immunohistochemical studies on sections of different human organs,

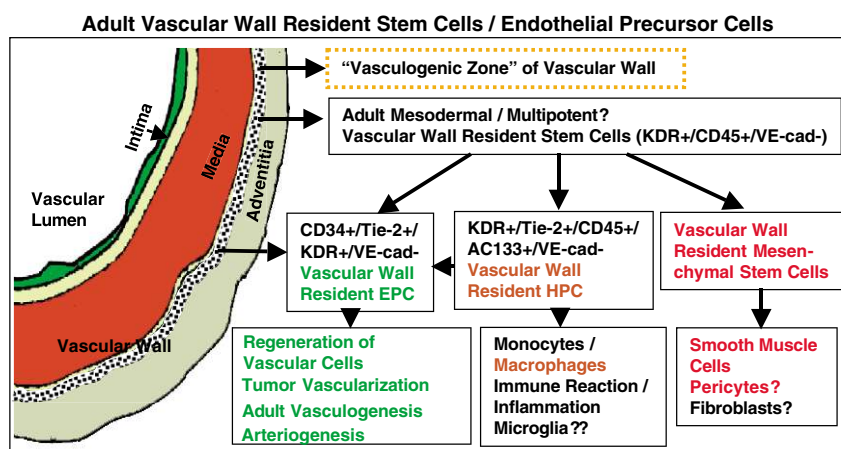


**Fig. 9. Recruitment of VW-EPC by tumor cells.** HITA rings co-cultured with cells of the human prostate cancer cell line DU-145 (A,B) show an extensive capillary outgrowth (arrows) into the lumen (A) and outside the rings (B) after 24 hours in culture. By contrast, no sprouts are seen in HITA rings cultured without tumor cells (C,D). Tumor cells (arrowheads) remain rounded and are not involved in the capillary outgrowth. Immunohistochemical studies on paraffin wax sections of several human organs show that CD34<sup>+</sup> cells (arrows) are present in the wall of large and mid-sized arteries (E) and veins (F), as demonstrated here exemplarily on sections of human prostate. By contrast, CD31 immunostaining is only detectable in mature endothelial cells lining the lumina of arteries (G) and veins (H).

including urinary bladder, testis, prostate, kidney, lung, heart, liver and brain disclosed the existence of vascular wall resident CD34<sup>+</sup> but CD31<sup>-</sup> cells in the same zone of the vessel as demonstrated in HITA. CD34<sup>+</sup> cells were found in mid-sized and large arteries and veins, e.g. human prostate blood vessels (9E-H).

## DISCUSSION

These data suggest the existence of VW-EPCs in human organs that serve as a local reservoir of cells for postnatal vasculogenesis. The present data also suggest the existence of a zone in the wall of large and mid-sized blood vessels that we termed 'vasculogenic zone' (Fig. 10). This zone is located at the border between the smooth muscle and the adventitial layer, and is to our knowledge the first reported location in the vascular wall of EPCs outside the bone marrow. Recently, the existence of vessel wall-derived endothelial progenitor cells was reported (Ingram et al., 2005) but no exact location of these cells within the vascular wall has been identified in



**Fig. 10. Hypothetical scheme of the 'vasculogenic zone'.** This scheme illustrates the concepts of the 'vasculogenic zone', which is defined as a vascular mural zone containing EPCs and probably also multipotent mesodermal stem cells here. Although the  $CD34^+/TIE2^+/KDR^+/VE-cadherin^-$  cells present in this zone differentiate into endothelial cells and form capillary-like sprouts from the vascular wall, the multipotent stem cells in this zone may serve as precursors of the in vitro and in vivo observed accumulation of macrophages. Obviously, they also have the capacity to differentiate into smooth muscle cells.

this study. The ex vivo marking of the cells of the vasculogenic zone by AdV5-GFP-system demonstrates that this zone of vascular wall serves a niche containing VW-EPC, which are capable of forming capillary sprouts. During this process, the cells became positive for endothelial markers and cell-adhesion molecules such as VEGF receptors, TIE2, VE-cadherin and CEACAM1, indicating their predetermination for endothelial cells. This interpretation is also supported by our findings showing the co-existence of CEACAM1 and CD34 in capillary-like sprouts from HITA-wall, as CEACAM1 is detectable in endothelial cells that are activated angiogenically or form new endothelial tubes, and in endothelial cells from AC133-positive progenitor cells (Ergun et al., 2000; Gehling et al., 2000).

The vasculogenic zone apparently contains cells of different subpopulations, which mainly consisted of EPCs and could be activated to form capillary sprouts in arterial ring assay in vitro. We named these cells vascular wall resident endothelial progenitor cells (VW-EPCs). During this activation there is a significantly increased accumulation of  $CD34^+$  cells not only in the vasculogenic zone but also in the whole adventitia of the vascular wall. While in the early stage of ring assay (within the first 6-8 days of culture) the  $CD34^+$  cells mainly migrate into the collagen gel outside the rings they transmigrate through the vascular wall towards the intima when the rings were cultured for longer than 14 days, and can also participate in capillary sprouting into the lumen of the rings. Further studies are needed to determine the possible role of the vasculogenic zone in the regeneration of vascular cells and/or formation of the *vasa vasorum* that supply the vascular wall with blood and nutrition.

A further interesting aspect of the VW-EPC is that they can be activated to formation of capillary sprouts by tumor cells as shown here in co-culture of the HITA rings with the prostate cancer cell line DU-145. Together with the findings here showing the presence of the  $CD34^+$  cells in the same location of vascular wall of arteries and veins of several organs, including prostate these results implicate a new view of local mechanisms providing tumor vascularization. Tumor cells can not only activate the pre-existing mature endothelial cells and C-EPCs but also EPCs residing in the wall of neighboring blood vessels that can apparently be easily acquired for the formation of new vessels. The better understanding of biology of these cells is necessary to understand the complex local processes that regulate tumor vascularization.

One of the cell populations we detected in the vasculogenic zone are the  $CD45^+$  cells. However, at this time it is not possible to determine exactly the source and the fate of the  $CD45^+$  cells residing in this zone of the vascular wall. It has been shown that a part of differentiating endothelial cells lining the embryonic aorta are

capable of differentiating into  $CD45^+$  cells and to serve as basis for hematopoiesis (Jaffredo et al., 1998). As we did not find a significant change in the number of  $CD45^+$  cells within the HITA wall after ring assay, we postulate that this may be due to their differentiation into macrophages or other hematopoietic cells. However, although  $CD34^+$  cells,  $CD105^+$  cells,  $VE-cadherin^+$  cells,  $occludin^+$  cells,  $KDR^+$  cells,  $TIE2^+$  cells and occasionally  $CD68^+$  cells were found to be involved in capillary sprouts from HITA, apparently no  $CD45^+$  cells participated in the capillary formation, although a few of them had migrated into the collagen gel outside the rings. These data are in line with previous findings showing that  $CD45^+$  cells originating from embryonic aortic endothelium became negative for endothelial markers once they expressed  $CD45$  (Jaffredo et al., 1998).

It has been shown that the migration and proliferation capacity of BM-EPCs decreases at higher ages (Dimmeler and Zeiher, 2004; Scheubel et al., 2003). In contrast to these circulating EPCs, the vascular wall resident EPCs of the vasculogenic zone are not directly exposed to shear stress, as they are distant from the luminal forces acting on mature endothelial and circulating EPCs. Moreover, this zone apparently contains multipotent stem cells that are capable of differentiating into macrophages but probably also into HPCs. These findings lead to the assumption that this vasculogenic zone of the vascular wall has not only the capability to serve as a source for vascular cells forming new blood vessels, but also to serve as a reservoir for inflammatory cells important for local immune response. As blood vessels containing this 'vasculogenic zone' are apparently present in all organs rather than being a unique phenomenon, we hypothesize that pre-existing blood vessels serve as basis for tumor vascularization not only by angiogenesis but also by postnatal vasculogenesis. Development of systems and techniques manipulating the function of these cells may be of therapeutic relevance for tissue vascularization as well as for anti-angiogenic tumor therapy.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/133/8/1543/DC1>

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