Only a specific subset of human peripheral blood monocytes has endothelial-like functional capacity

Elzafir Elsheikh MSc⁴, Mehmet Uzunel PhD², Zhong He MD², Jan Holgersson MD, PhD³, Grzegorz Nowak MD, PhD¹, and Suchitra Sumitran-Holgersson PhD¹

Divisions of ¹Transplantation Surgery and ²Clinical Immunology, Karolinska Institutet, Karolinska University Hospital-Huddinge, S-141 86 Stockholm. Sweden.

Total word count: 4581

Subject heading: Hemostasis, Thrombosis, and Vascular Biology

Address correspondence to: Dr. Suchitra Sumitran-Holgersson,
Department of Transplantation Surgery B56,
Huddinge University Hospital,
Tel: + 46 8 58583988.
Fax: + 46 8 58581390.
E-mail: suchitra.holgersson@cfss.ki.se.
Abstract

The monocyte population in blood is considered as a possible source of endothelial precursors. Since endothelial-specific receptor tyrosine kinases act as regulators of endothelial cell functions, we investigated whether the expression of the vascular endothelial growth factor receptor-2 (VEGFR-2) on monocytes is important for their endothelial-like functional capacity. Peripheral blood monocytes expressing VEGFR-2 (CD14+/VEGFR-2+) were isolated and their phenotypic, morphological and functional capacity was compared with monocytes negative for this marker (CD14+/VEGFR-2-). CD14+/VEGFR-2+ cells constituted approximately 2.0±0.5% of the total population of monocytes and 0.08±0.04% of mononuclear cells in blood.

CD14+/VEGFR-2+ cells exhibited the potential to differentiate in vitro into cells with endothelial characteristics. The cells were efficiently transduced by a lentiviral vector driving the expression of the green fluorescence protein (GFP). Transplantation of GFP-transduced cells into balloon-injured femoral arteries of nude mice significantly contributed to efficient re-endothelialization. CD14+/VEGFR-2- did not exhibit any of the above characteristics. These data demonstrate that expression of VEGFR-2 on peripheral blood monocytes is essential for their endothelial-like functional capacity, and supports the notion of a common precursor for monocytic and endothelial cell lineage. Our results help in clarifying which specific subpopulations may restore damaged endothelium and participate in the maintenance of vascular homeostasis.
Angiogenesis is a process of new blood vessel development (neovascularization) from pre-existing vasculature, while vasculogenesis refers to blood vessel formation from endothelial progenitors that differentiate in situ. Until recently, angiogenesis was considered the only means of adult neovascularization and vasculogenesis was thought to be limited to embryological development. However, the existence of circulating endothelial progenitor cells (EPCs) has provided evidence that postnatal vasculogenesis also occurs in adults. The potential of EPC as therapeutic tools for rescue of tissues from ischemic damage has been suggested. Among the number of different factors implicated in the regulation of the angiogenic response, vascular endothelial growth factor (VEGF), an endothelial cell specific mitogen and a potent inducer of vascular permeability, is perhaps the most important player.

VEGF acts through specific tyrosine kinase receptors that includes VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR) and VEGFR-3/Flt-4 which convey signals that are essential for embryonic angiogenesis and hematopoiesis. While VEGF binds to all three receptors, most biological functions are mediated via VEGFR-2 and the role of VEGFR-1 is currently unknown. VEGFR3/Flt4 signaling is known to be important for the development of lymphatic endothelial cells and VEGFR3 signaling may confer lymphatic endothelial-like phenotypes to endothelial cells. VEGFRs relay signals for processes essential in stimulation of vessel growth, vasorelaxation, induction of vascular permeability, endothelial cell migration, proliferation and survival. Endothelial cells express all different VEGF-Rs. During embryogenesis, it has been reported that a single progenitor cell, the hemangioblast can give rise to both the hematopoietic and vascular systems. Work addressing the origin of endothelial progenitor lineage in adult peripheral blood has demonstrated that monocytes also co-express endothelial lineage markers such as VEGFR-2 and Ac133, and have the capacity to differentiate into adherent mature
endothelial cells and form cord-like structures in matrigel.\textsuperscript{10,11} Rehman et al recently reported that peripheral blood endothelial-like cells are derived from monocytes/macrophages and secrete angiogenic growth factors.\textsuperscript{12} Furthermore, S. Dimmeler’s group demonstrated that both CD14\textsuperscript{+} and CD14\textsuperscript{-} populations in peripheral blood have the capacity to improve neovascularization after hind-limb ischemia.\textsuperscript{13}

Since VEGFR-2 is an endothelial-specific receptor that controls many aspects of vascular growth and angiogenic responses\textsuperscript{14,15} we hypothesised that expression of this endothelial-specific tyrosine kinase receptor on CD14\textsuperscript{+} monocytes may be important for their ability to function as EPC. We therefore isolated circulating monocytes with and without expression of this receptor and studied their functional capacity including their ability to contribute to re-endothelialization in balloon-injured femoral arteries of nude mice.
Methods

The present study was approved by the local ethics committee.

**Isolation of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells from peripheral blood**

Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation with Ficoll from blood of healthy human volunteers according to standard procedure. Approval was obtained from the Karolinska University Hospital institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki. CD14+/VEGFR-2+ cells were isolated by fluorescence-activated cell sorting. Staining was carried out as follows: PBMC were initially incubated with anti-CD3 antibody coated magnetic particles (DYNAL, Norway) to remove T lymphocytes. The procedure was carried out as described by the manufacturers. The remaining cells were distributed into several tubes each containing 20 x 10⁶ cells. 15 μl of anti-VEGFR-2 (20 μg/ml, Reliatech, Germany) antibodies were added to each tube and incubated for 30 min at room temperature. After washing once with phosphate buffered saline (PBS) the cells were labeled with 20 μl of a 1:10 diluted secondary FITC-conjugated goat anti-mouse monoclonal antibody (Serotec, Sweden) and 20 μl PE-conjugated anti-CD14 antibodies (4μg/ml, BD Biosciences, USA). During cell sorting, using a stringent gate, highly purified CD14+/VEGFR-2+ as well as the CD14+/VEGFR-2- fractions were collected for further analysis.

**Western blotting**

Lysates of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells (1x10⁶ cells for each population) were separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with...
anti-VEGFR-2 antibodies (1:200). Horseradish peroxidase conjugated F(ab’)2 fragments of goat anti-mouse secondary antibodies (1:2000) (Jackson Immunoresearch, USA) were used together with the ECL™ kit (Amersham) to identify the bound antibody.

Phenotyping and cell cultivation

FACS sorted cells were placed on glass slides using cytospin. Immunocytochemistry was performed by enzyme staining. The cells were stained with the following antibodies: anti-CD133, -CD141 (Thrombomodulin), -CD144 (VE-Cadherin), all from Becton Dickinson, USA, -von Willebrand Factor (Serotec, England) and antibodies to endothelial nitric oxide synthase (e-NOS) (all antibodies were used at a dilution of 1:50) for 1 hour at room temperature, washed three times with PBS and stained with secondary biotinylated goat anti-mouse antibodies (1:500). The avidin-peroxidase procedure was carried out using Vectastain Elite ABC kit (ImmunKemi, Stockholm, Sweden) as described by the manufacturers. The ACE (gives red staining) or DAB-Nickel (gives brown/black staining) substrate kits were used as color developers. The two populations were further characterized by flow cytometry as described earlier using an array of antibodies to specific markers expressed on monocytes and endothelial cells (Table 1). FITC conjugated-acetylated low density lipoprotein (AcLDL) (Molecular Probes,USA), antibodies to-von Willebrand factor (DAKOPATTS, Denmark), α-actin, fibroblast (Serotec, Sweden), and the Ulex europaeus (Sigma, Sweden) were used. All other antibodies were purchased from Becton Dickinson (USA). Corresponding control isotypes were used for evaluation of non-specific binding of monoclonal antibodies (Mabs). The cells were analyzed on a Becton Dickinson flow cytometer (FACSorter).
For some experiments, sorted CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells were cultivated on fibronectin (20 µg/ml)–coated tissue culture plates, in endothelial selective medium EndoCult™ (Stem Cell Technologies, Canada). These cultures were observed for growth and morphology. The proliferative capacity of the two cell types was tested by addition of BrdU. The assay was performed as described earlier. The cells after one week in culture were cytopspinned on glass and stained for VE-Cadherin expression.

Human aortic endothelial cells (HAECs) were purchased from Clonetics and cultivated in recommended medium. HAECs were used as controls in the phenotypic analysis and the tubule formation assay.

**Transmission electron microscopy**

Electron microscopic analysis was performed at the core facility unit for electron microscopy at Karolinska University hospital-Huddinge, Sweden. CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells were grown separately on membrane filters in 24 well plates. The membranes were fixed in 2% glutaraldehyde, briefly rinsed in distilled water, placed in 70% ethanol for 10 min, and in 99.5% ethanol for 15 min, all at 4°C and dried. After fixation, the membrane was cut free, fixed for 1 hr at 4°C in a buffer containing 0.15 M sodium cacodylate, 1% osmium tetraoxide, and 3 mM CaCl₂, pH 7.4. Subsequently, the wells were rinsed briefly, in 0.15 M sodium cacodylate buffer, dehydrated in ethanol as described above, and imbedded in Spurr resin (Agar Scientific LTD, Essex, England). The sections were contrasted with uranyl acetate followed by lead citrate, and examined at 80 kV in a Leo 906 (Oberkochen, Germany) transmission electron microscope.

**Migration assay**
Freshly isolated CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells (5 x 10⁴) were added to four fibronectin-coated Transwell inserts each, and all having a 3.0 µm membrane pore size. The cell migration were assayed in the absence and presence of 50 ng/ml in the lower chamber of vascular endothelial growth factor (VEGF) or 300 ng/ml monocyte chemotactic factor-1 (MCP-1) in medium containing 5% fetal calf serum. Cells were allowed to migrate for 22 hrs. Migrated cells were collected and counted in a haemocytometer. Each experiment was performed in triplicate wells and repeated four times.

**Angiogenic growth factor secretion by CD14+/VEGFR-2+ and CD14+/VEGFR-2- cell populations**

To assess growth factor secretion, freshly isolated cells were cultivated in growth factor- and serum-free medium for 72 hrs. Supernatants from all cultures were collected and stored at −70°C until further analysis. Conditioned medium were assayed for the angiogenic growth factors, VEGF, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF) and stromal derived-factor-1 (SDF-1). The growth factors were assayed by ELISA (R&D systems).

**In vitro angiogenesis assay**

The formation of capillary tube-like structures by freshly isolated CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells was assessed in a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma, frequently used for the evaluation of *in vitro* angiogenesis. Twenty-four well plates were coated with 200 µl of Matrigel (pregelled for 30 minutes at 37 °C in 5% CO₂) and the cells were seeded on the polymerized matrix at a density of
5x10^4 cells/well. Resulting tube-like structures were examined using a phase-contrast light microscope after cells were cultivated for one week at 37° C in 5% CO2. HAECs were used as positive control cells.

**Lentiviral transduction of FACS sorted CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells**

The recombinant lentiviral vector was produced using a three-plasmid expression system as described earlier. For transduction, FACS sorted cells were transferred to 10 ml tubes with 1 ml culture medium and incubated with concentrated pHR’EF1-GFP SIN virions at a MOI of 0.1 at 37 °C in a 5% CO2 atmosphere overnight. After virus incubation, cells were washed once in PBS and directly injected in the denuded femoral artery of nude mice (see below). To detect GFP expression, following the same transduction procedure, transduced cells were cultured in six-well plate and observed on day six post-transduction by fluorescent microscopy.

**Mice**

Balloon injury of the right femoral artery was performed as follows: nude C57 black mice (Taconic M&B, Denmark) with body weight of 20-25 g were anesthetized with isofluorane, and the right femoral artery was exposed to the level of bifurcation through a trans-abdominal incision. Microclamps (S&T, Switzerland) were placed on lower aorta, left iliac artery and the distal part of the right femoral artery. A 2F Fogarty balloon catheter (Baxter) was introduced into the right femoral artery, inflated, and withdrawn three times with rotation. Inflation was performed via the cannula and 1ml of Ringer solution with free outflow through the micro-incision. Cells (5 x 10^5 cells per animal) in 100 µl MCDB medium (CD14+/VEGFR-2+, n=10; CD14+/VEGFR-2-, n=10), were instilled through the same cannula and incubated in the freshly
injured arterial bed for 15–20 min while control-transplanted mice received only culture medium (mice, n=6). After incubation, unbound cells were aspirated, the catheter was removed, and the micro-incision was sutured with 11-0 nylon (S&T, Switzerland) interrupted suture. By removing the micro-clamps the blood flow was restored. All animal procedures were performed in accordance with institutional guidelines and conformed to the guide for the Care and Use of Laboratory Animals at Huddinge University hospital in Sweden. Animals were sacrificed at 2 and 4 weeks after human cell transplantation and the right femoral artery was harvested for histopathological examinations. Vessels were embedded in O.C.T. and frozen in liquid nitrogen.

**Immunohistochemistry**

To localize human cells in the mouse artery, 5-µm cryosections were stained with an anti-GFP antibody (1:50). The immunoperoxidase procedure was carried out using Vectastain Elite ABC kit (ImmunKemi, Stockholm, Sweden) as described by the manufacturers. The ACE (gives red staining) substrate kit was used as color developer and counter-stained with hematoxylin. In addition, a mouse anti-human nuclei monoclonal antibody (Chemicon, CA, USA) was used to detect human cells in the mouse artery by immunofluorescence. The phenotype of the transplanted human cells was detected by double staining with antibodies to human nuclei (1:50), and anti-VE-Cadherin (1:100). This was followed by staining with secondary anti-mouse subclass-specific FITC or Texas red conjugated antibodies (1:500). Endothelialization was calculated as the percentage of the surface covered by human ACE positive cells and the total luminal surface. For histomorphology (six animals per group, 6 sections/femoral artery) cross-sections of the artery were stained with hematoxylin/eosin and examined for vessel diameter, media area, and intima to media area ratio.
Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from three human-mouse chimeric femoral arteries, one mouse receiving CD14+/VEGFR-2- cells and one sham transplanted mouse at 4 weeks after transplantation, using the Micro-FastTrack RNA isolation kit (Invitrogen, Groningen, The Netherlands). Human specific primers were used to detect human CD31 and e-NOS in the human cell-transplanted mice arteries. Freshly isolated CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells were also tested for expression of e-NOS. ABL was used as an endogenous reference gene. Primer sets were commercially synthesized by CyberGene (Huddinge, Sweden). Primer sequences were

“CD31-F” : 5’-CCA CTG CAG AGT ACC AGG TGT TGG-3’,
“e-NOS-F” : 5’-CTG TAT GGC TCC GAG ACC-3’
“e-NOS-R” : 5’-GCT GTT GAA GCG GAT CTT ATA AC-3’, expected product size (bp):279
“ABL-F” : 5’-CGG CTC TCG GAG GAG ACG ATG A-3’,
“ABL-R” : 5’-CCC AAC CTT TTC GTT GCA CTG T-3’, expected product size (bp):385.

The PCR reaction was carried out as described earlier.19

Statistical analysis

Where applicable, results are presented as mean ± SD. Mann-Whitney U test was used for comparisons between the groups. p<0.05 was considered to be significant.
Results

Characterization of CD14+/VEGFR-2+ and CD14+/VEGFR-2-

We found that CD14+/VEGFR-2+ cells constitute approximately 2±0.5%, (Fig. 1A) of the total population of monocytes and 0.08±0.04% of PBMCs in blood. Immunocytochemical analysis demonstrated that the freshly isolated CD14+/VEGFR-2- cell fraction did not express e-NOS (Fig. 1B). However, freshly isolated CD14+/VEGFR-2+ cells expressed e-NOS, von Willebrand Factor, thrombomodulin and some cells expressed CD133 (Fig. 1C) but not VE-Cadherin. Expression of the VEGFR-2 receptor on CD14+/VEGFR-2+ cells was further supported by the fact that, lysates of this population when immunoblotted with anti-VEGFR-2 antibodies gave the expected band of approximately 200 kDa which was absent or faintly expressed in the CD14+/VEGFR-2- cell population (Fig. 1D). Furthermore the e-NOS mRNA was strongly expressed in CD14+/VEGFR-2+ cells, while in the VEGFR-2 negative fraction only a faint e-NOS mRNA band was visible (Fig. 1E). We cannot rule out the fact that this faint band could be due to a small number of contaminating VEGFR-2+ cells in the CD14+/VEGFR-2- cell population. The phenotypic markers expressed by the two populations are shown in Table 1. Both populations expressed the CD45 marker indicating the hematopoietic origin of these cells.

Electron microscopic analysis of CD14+/VEGFR-2+ cells cultured on Transwell tissue culture inserts demonstrated formation of a basement membrane, the presence of Weibel Palade bodies and tight junctions characteristic of endothelial cells (Fig.2A-C), while the negative fraction did not exhibit these features.
CD14+/VEGFR-2+ cells when cultivated on fibronectin-coated culture wells formed colonies after three days in culture. These colonies over the course of the next few days changed morphology from a center of densely packed round cells to seemingly mature flattened, elongated cells. On the other hand, CD14+/VEGFR-2- population remained adherent as slightly rounded cells for two weeks but did not form clusters (Fig. 2D). Cultivated CD14+/VEGFR-2+ cells expressed VE-Cadherin after 1 week in culture (Fig. 2D), while the VEGFR-2- fraction did not express this marker at any time point during cultivation. Significantly higher numbers of BrdU+ cells were observed in the CD14+/VEGFR-2+ cell population as compared to the CD14+/VEGFR-2- population (Fig. 2E), indicating higher proliferative capacity in the CD14+/VEGFR-2+ cell population. Nevertheless, this population could be subcultured for only 4-5 passages.

**In vitro** functional capacity of CD14+/VEGFR-2+ and CD14+/VEGFR-2- peripheral blood cells

Significantly higher numbers of freshly isolated CD14+/VEGFR-2+ cells migrated towards VEGF and MCP-1 as compared with CD14+/VEGFR-2- cells (Fig. 3A) (p=0.009 and p=0.047 respectively). The angiogenic growth factors produced by the two populations did not vary significantly. Both populations produced VEGF, G-CSF and GM-CSF but not SDF-1 or FGF. However, growth factor levels were higher in the CD14+/VEGFR-2+ population as compared to the CD14+/VEGFR-2- population (Fig. 3B). In matrigel, three-days cultivated cells showed that only CD14+/VEGFR-2+ but not CD14+/VEGFR-2- cells could form tubule-like structures (Fig. 3C). HAECs were used as controls.

Thus, the *in vitro* data indicated that, freshly isolated CD14+/VEGFR-2+ cells demonstrated endothelial-like characteristics as compared to CD14+/VEGFR-2- cells.
CD14+/VEGFR-2+ cells differentiated to endothelial cells and efficiently repopulated denuded mouse femoral arteries

Exposure of the two cell populations to lentiviral particles expressing GFP led consistently to transduction efficiencies of >70%. Screening of artery sections for human cells demonstrated that due to high auto-fluorescence, visualisation of GFP+ cells was difficult. We therefore performed an enzyme-based immunohistochemical staining using anti-GFP antibody to detect GFP+ cells. Using this antibody we found no GFP-positive areas in sham-transplanted and CD14+/VEGFR-2-transplanted mice (Fig. 4A-C). However, coverage of the intimal surface of arteries with GFP-positive cells (red) was seen already at 2 weeks after transplantation in mice receiving CD14+/VEGFR-2+ cells (Fig. 4D). The GFP-positive area was significantly increased in vessels harvested 4 weeks after seeding (Fig. 4E&F). In addition, human cells were also identified by an anti-human nuclei antibody using immunofluorescence. The specificity of the human-nuclei antibody was first determined by using a normal human and mouse artery. Human cell-nuclei stained red in color (Fig. 5A). Control mouse artery did not stain with the human nuclei antibody (Fig. 5B). At 4 weeks post-transplantation a high degree of repopulation was seen and approximately 70±8% of the lesion was covered with human cells (red) in mice receiving CD14+/VEGFR-2+ human cells (Fig. 5C) but not in mice transplanted with CD14+/VEGFR-2-cells (Fig.5D). Animals transplanted with CD14+/VEGFR-2- cells did not demonstrate the presence of these cells in the denuded arteries, spleen, lungs or in circulation at 2 or 4 weeks after transplantation (data not shown). Endothelial cell phenotype in vivo was confirmed by staining
with antibodies to VE-Cadherin (green) and each section was double-stained with the anti-human nuclei antibody to detect human cells (red). The double stained cells are stained yellow (Fig. 5E). Cross-sections of injured arterial segments were examined at 2 and 4 weeks post transplantation for histomorphological changes. The balloon-injury procedure in the mouse femoral artery resulted in dilation of the lesioned arterial segment so that the inner vessel diameter was significantly increased from $0.301 \pm 0.041$ mm in uninjured control arteries to $0.601 \pm 0.043$ and $0.631 \pm 0.028$ mm in injured vessels that were incubated with media alone or CD14+/VEGFR-2- respectively ($p<0.001$). This widening was not found in femoral arteries of mice $0.404 \pm 0.08$ mm that were transplanted with CD14+/VEGFR-2+ cells. A reduction in cross-sectional media area from $0.028 \pm 0.010$ to $0.012 \pm 0.007$ mm$^2$ was observed in injured arteries. Thinning out of media area was not seen in CD14+/VEGFR-2+ cell transplanted arteries $0.033 \pm 0.013$. Re-endothelialization mediated by these cells did not result in neointima formation in femoral arteries at 2 or 4 weeks post injury. The intimal to media area ratio was not significantly higher in CD14+/VEGFR-2+ transplanted vessel segments (I/M ratio: normal uninjured artery $0.03 \pm 0.009$, media control $0.02 \pm 0.008$, CD14+/VEGFR-2- $0.02 \pm 0.009$; CD14+/VEGFR-2+ $0.04 \pm 0.02$, $p=ns$).

Transcription of human endothelial-specific genes in mice transplanted with CD14+/VEGFR-2+ cells

We further confirmed the engraftment of the transplanted cells by determining the expression of human genes in transplanted mice. We analysed femoral arteries of the mice sacrificed one month after transplantation with human cells by RT-PCR using primers specific for the human endothelial-specific gene CD31 and e-NOS. The CD31 and e-NOS primers were species specific
for human as they did not amplify the respective mouse gene (Fig. 6A). These results were obtained with arteries from mice receiving CD14+/VEGFR2+ cells, but not in those transplanted with CD14+/VEGFR-2- cells. These data demonstrate that the transplanted human CD14+/VEGFR-2+ cells engraft the damaged mouse artery.
Controversy exists with respect to the identification and the origin of EPCs. EPC and endothelial-like cell populations can be grown from cells expressing hematopoietic markers²²⁰,²¹ myeloid cells¹⁰,¹²,¹³,²² “side population” cells²³ and circulating mature endothelial cells.²⁴ Thus, the circulating cell population that contributes to postnatal neovascularization is heterogenous and displays variable morphological growth characteristics. Moreover, within these populations it may only be a small specific fraction of the cells that have this capacity. Prior studies have demonstrated that circulating monocytes can develop an endothelial phenotype¹⁰,²² and have the ability to take part in neovascularization.¹³ A recent study demonstrated that peripheral blood endothelial-like cells are derived from monocytes/macrophages.¹² Published results indicate that it is only a subpopulation of monocytes that may have endothelial-like characteristics. Thus, although there is convincing evidence that endothelial-like cells generated from monocytic lineage improve neovascularization, the exact phenotype and characterization of the monocyte subpopulation capable of generating endothelial-like cells is not known.

In the present study, we demonstrate that circulating CD14+ monocytes that express the endothelial specific tyrosine kinase receptor VEGFR-2 have EPC capacity, whereas CD14+ cells not expressing this marker lacked this capacity. The CD14+/VEGFR-2+ cell population demonstrated features required by cells involved in revascularization. This population demonstrated the ability to migrate towards angiogenic growth factors VEGF or MCP-1, formed capillary-like tubules, and demonstrated presence of Weibel-Palade bodies, tight junctions, and production of basement membrane. These cells responded to vascular injury by restoring the endothelial lining of damaged arteries within 2-4 weeks. This finding may have implications for treatment of vascular proliferative disease, since restenosis after balloon angioplasty remains a
major problem in vascular therapy. *Ex vivo* expansion of these cells may have implications for cell-based therapies for vascular diseases. Our results showed that under the growth culture conditions used in the present study, this population demonstrated limited proliferative capacity *in vitro*, albeit higher than the CD14+/VEGFR-2- population. We are currently working out conditions that may allow even greater expansion of these cells *in vitro*. It is likely that these cells *in vivo* may attain very proliferative capacity, since the balloon-injury may provide a microenvironment that is conducive for proliferation of EPC by producing several factors including growth factors, cytokines and other proteins. EPC are defined as cells with extensive clonogenic and proliferative capacity lacking expression of CD45 and CD14.25,26 On the other hand, since CD14+/VEGFR-2+ cells express CD45 with limited proliferative capacity, the possibility remains that they may be terminally differentiated cells and not progenitor cells. Our continued on going studies using this population will help answer some of these questions in the future. Nonetheless, the above observations indicate that circulating CD14+/VEGFR-2+ cells can be recruited to sites of vascular damage and may take part in re-endothelialization during vascular injury.

On the other hand, CD14+/VEGFR-2- population did not demonstrate any of the above functional properties. They exhibited the classical monocytic phenotype and expressed high levels of the VEGFR-1 marker. Thus, although CD14+/VEGFR-2- cells expressed VEGFR-1 which is also a high-affinity tyrosine kinase receptor for VEGF, they did not demonstrate high migratory capacity towards VEGF and did not take part in re-endothelialization. Expression of VEGFR-1 though considered to be restricted to endothelial cells has been found on monocytes and macrophages.27 Furthermore, this population expressed little or no e-NOS. e-NOS is essential for neovascularization.28 It is not only important in mobilization of stem and progenitor cells but
also determines their angiogenic capacity in ischemic tissue. e-NOS-deficient cells are impeded by a marked reduction in homing and incorporation into microvessels. Thus, the lack of VEGFR-2 receptor and e-NOS expression on the CD14+/VEGFR-2- cells and the weak signalling abilities of VEGFR-1 may contribute to the “non-endothelial character” and ineffectiveness of the CD14+/VEGFR-2- cells in the process of re-endothelialization.

Both the populations CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells were amenable to transduction by lentiviral vectors with high efficiency, thus implicating their potential use as vehicles for delivery of therapeutic genes at site of injury. However, it was only the CD14+/VEGFR-2+ cells that engrafted and were found in the seeded grafts and therefore this may be a good candidate population for therapeutic applications. The CD14+/VEGFR-2- cells were not detected at the site of injury or in other organs at 2 or 4 weeks after transplantation. We are currently performing additional work to follow the fate of these cells immediately and upto 1 week after transplantation.

A common progenitor to endothelial and smooth muscle cells has been proposed. Studies have demonstrated the presence of functional VEGF receptors (VEGFR-2 and VEGFR-4) on vascular smooth muscle cell. Unlike our previous report where VEGFR-2+ cells differentiated into both endothelial and smooth muscle cells in vivo, in the present study CD14+/VEGFR-2+ after transplantation were found only in the luminal surface of the seeded arteries but not in the media. This indicates that a different subpopulation of cells present in blood expressing VEGFR-2 may be precursors of smooth muscle cells.
Data suggest that monocytic cells are necessary for arteriogenesis and possibly neovascularization.\textsuperscript{31,32} However, until now a possible direct role for these cells in the re-endothelialization process has not been reported. Re-endothelialization with CD14+/VEGFR-2+ cells restored vessel diameter and media thickness in balloon-injured mice femoral arteries. Furthermore no neointima formation was observed in animals transplanted with these cells. It is likely that the efficiency of re-endothelialization may not solely be attributable to the incorporation of these cells in the denuded arteries, but may also be influenced by the release of proangiogenic factors. Thus, the CD14+/VEGFR-2+ population may improve re-endothelialization by production of the growth factors. Indeed CD14+/VEGFR-2+ cells in cultivation produced higher levels of VEGF, G-CSF and GM-CSF as compared to CD14+/VEGFR-2- cells. In all studies so far using CD14+ monocytes as the starting population to generate EPCs, the investigators have first generated these cells \textit{in vitro} to endothelial cells by cultivation in medium favouring endothelial differentiation. However, in this study we demonstrate that there exists a subpopulation of monocytes in the circulation that already express many endothelial cell markers and therefore this population may be recruited during re-endothelialization. This might be the most likely population that is recruited to regenerate low grade endothelial damage induced by risk factors for coronary artery disease.\textsuperscript{30} Thus this population might have an important role as an endogenous repair mechanism to maintain the integrity of the endothelial monolayer by replacing denuded parts of the artery.

In summary, we report a novel finding that the expression of the tyrosine kinase receptor VEGFR-2 on CD14+ monocytes is important for their endothelial-like functional capacity. Freshly isolated circulating CD14+/VEGFR-2+ cells when transplanted, exhibited functional competence to improve re-endothelialization in balloon-injured femoral arteries. Thus by
restoring an intact endothelium, these cells might participate in the maintenance of vascular homeostasis.
Acknowledgments

The present study was financed by grants from the Lars Erik Gelins Foundation and The Swedish Research Council no. K2002-06X-14004-02B to Dr. Sumitran-Holgersson.

No conflict of interest exist.
References


### Table 1

Phenotypic characteristics of freshly isolated CD14+/VEGFR-2- cells from peripheral blood

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>CD14+/VEGFR-2+ cells (n=4) %</th>
<th>CD14+/VEGFR-2-cells (n=5) %</th>
<th>HAECs % (primary cell line)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stem/progenitor/mature endothelial cell markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>100</td>
<td>0.005 ± 0.001</td>
<td>5</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>78 ± 1.5</td>
<td>81 ± 1.33</td>
<td>3</td>
</tr>
<tr>
<td>Tie-2</td>
<td>100</td>
<td>23 ± 2.5</td>
<td>0</td>
</tr>
<tr>
<td>CD34</td>
<td>3 ± 2.1</td>
<td>0.01 ± 0.007</td>
<td>2</td>
</tr>
<tr>
<td>CD123</td>
<td>0</td>
<td>28 ± 2.1</td>
<td>0</td>
</tr>
<tr>
<td>CD133</td>
<td>2± 0.6</td>
<td>0.05 ± 0.002</td>
<td>0</td>
</tr>
<tr>
<td>CD90</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AcLDL</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CD31</td>
<td>100</td>
<td>20 ± 2.6</td>
<td>100</td>
</tr>
<tr>
<td>CD144</td>
<td>0.2 ± 0.01</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>vWF</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><strong>Ulex europaeus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>Monocyte/macrophage markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>98 ± 0.2</td>
<td>97 ± 2.2</td>
<td>0</td>
</tr>
<tr>
<td>CD15</td>
<td>9 ± 0.02</td>
<td>8 ± 3.2</td>
<td>0</td>
</tr>
<tr>
<td>CD11b</td>
<td>95 ± 7.2</td>
<td>92 ± 2.4</td>
<td>0</td>
</tr>
<tr>
<td>CD11c</td>
<td>6 ± 5.6</td>
<td>84 ± 2.7</td>
<td>0</td>
</tr>
<tr>
<td>CD68</td>
<td>0</td>
<td>3 ± 1.5</td>
<td>0</td>
</tr>
<tr>
<td>CD83</td>
<td>0</td>
<td>2.7 ± 1.4</td>
<td>0</td>
</tr>
<tr>
<td><strong>Control markers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alpha-actin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD45</td>
<td>78 ± 10.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>CD3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD56</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD16</td>
<td>95 ± 4.8</td>
<td>92 ± 0.4</td>
<td>0</td>
</tr>
<tr>
<td>CD19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SD
Figure Legends

**Figure 1. Characterization of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells.** A, Flow cytometry sorting of CD14+/VEGFR-2+ and CD14+/VEGFR-2- from peripheral blood mononuclear cells depleted of T lymphocytes. B, CD14+/VEGFR-2- cells did not express endothelial nitric oxide syntahse (e-NOS). C, CD14+/VEGFR-2+ cells expressed several endothelial-specific markers such as e-NOS (Black), von WilleBrand Factor (red) and thrombomodulin (red) and some cells expressed CD133 (red) but not CD144. The cells were counterstained with hematoxylin. Magnification 40x. D, Western blotting of the lysates from CD14+/VEGFR-2+ cells with antibodies to VEGFR-2 gave the expected bands of app. 200kDa (Lanes 1&2), while CD14+/VEGFR-2- cells (Lane 3) and the control secondary antibody did not (Lane 4). E, A strong mRNA expression for e-NOS was detected in CD14+/VEGFR-2+ cells and weakly in the CD14+/VEGFR-2- cells.

**Figure 2. In vitro** morphological characterization of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells. A, TEM micrographs illustrate that CD14+/VEGFR-2+ cells demonstrated the presence of tight junctions typical for endothelial cells (arrows, bar; 2µm), B, Weibel-Palade bodies (arrow, bar; 0.5µm) and C, produced basement membrane (M, bar; 1µm). D, CD14+/VEGFR-2- cells in culture did not form colonies while the CD14+/VEGFR-2+ cells formed colonies consisting of rounded cells initially which after one week grew out as single elongated cells. After one week in culture only the CD14+/VEGFR-2+ cells expressed VE-Cadherin (red). E, Significantly higher numbers of CD14+/VEGFR-2+ cells were BrdU+ as compared to CD14+/VEGFR-2- cells.
**Figure 3.** *In vitro* migratory and tubule-forming capacity of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells. A, In a migration assay in the absence and presence of 50 ng/ml concentration of vascular endothelial growth factor (VEGF) and 300 ng/ml monocyte chemotactic protein-1 (MCP-1) a significantly higher number of CD14+/VEGFR-2+ cells migrated towards VEGF and MCP-1 as compared to CD14+/VEGFR-2- cells. B, CD14+/VEGFR-2+ cell population produced higher levels of the angiogenic growth factors, VEGF, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) as compared to the CD14+/VEGFR-2- population. C, CD14+/VEGFR-2+ but not CD14+/VEGFR-2- cells formed capillary-like tubules in matrigel. Human aortic cells (HAECs) were used as control cells.

**Figure 4.** *In vivo* engraftment capacity of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells. Immunohistochemical staining of sections from balloon-injured mouse femoral artery with an anti-green fluorescence protein (GFP) antibody showed A, no localisation of GFP-positive areas in sham-transplanted and B, CD14+/VEGFR-2-transplanted cells in the luminal surface of the seeded artery. C, section showing staining with control secondary antibody. D, a significant number of GFP-positive (red) CD14+/VEGFR-2+ cells (arrows) at 2 weeks and E&F, at 4 weeks were found in the animals transplanted with these cells. Original magnification 60x.

**Figure 5.** Immunofluorescence staining for detection of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells after transplantation. The human-nuclei antibody stained cells (red) in the A, normal human artery, B, but not control injured sham-transplanted mouse artery. C, Balloon-injured mouse femoral artery showed the localization of transplanted human CD14+/VEGFR-2+ cells in the luminal surface of the seeded artery (red cells, arrows) at 4 weeks.
However, no localization of transplanted CD14+/VEGFR-2- cells was observed at 4 weeks. E, The endothelial cell phenotype of transplanted human cells was confirmed by double staining with human-nuclei antibody and antibodies to VE-Cadherin (double positive cells are stained yellow, arrows) Original magnification 60x.

**Figure 6. In vivo engraftment and functional capacity of CD14+/VEGFR-2+ and CD14+/VEGFR-2- cells.** A, Transcription of human endothelial-specific genes in the mouse artery. Human CD31 and e-NOS were detected in balloon-injured mouse femoral arteries that received human CD14+/VEGFR-2+ cells but not in the sham or CD14+/VEGFR-2- cell transplanted mice. ABL was used as the house-keeping gene.
Figure 4
Figure 5
Figure 6

<table>
<thead>
<tr>
<th>Marker</th>
<th>Human control</th>
<th>Shear filament</th>
<th>CDH1-VEGFR-2- N mutant no.1</th>
<th>CDH1-VEGFR-2- N mutant no.2</th>
<th>CDH1-VEGFR-2- N mutant no.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH1</td>
<td>248 bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e-NOS</td>
<td>279 bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABL</td>
<td>385 bp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>