Characterization of Human Late Outgrowth Endothelial Progenitor-Derived Cells under Various Flow Conditions

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Key Words

Endothelial cell • Tissue factor • Shear stress

Abstract

Background: Endothelial progenitor-derived cells (EPC) are a cell therapy tool in peripheral arterial disease and for re-endothelialization of bypasses and stents. **Objective:** To assess EPC behavior under flow conditions normally found in vivo. **Results:** EPC were isolated from human cord blood, cultured on compliant tubes and exposed in an in vitro flow system mimicking hemodynamic environments normally found in medium and large arteries. EPC exposed for 24 h to unidirectional (0.3 ± 0.1 or 6 ± 3 dynes/cm²) shear stress oriented along flow direction, while those exposed to bidirectional shear stress (0.3 ± 3 dynes/cm²) or static conditions had random orientation. Under bidirectional flow, tissue factor (TF) activity and mRNA expression were significantly increased (2.5- and 7.0-fold) compared to static conditions. Under low shear unidirectional flow TF mRNA increased 4.9 ± 0.5 -fold. Similar flow-induced increases were observed for TF in mature umbilical vein-derived endothelial cells. Expression of tissue-type plasminogen activator (t-PA), urokinase (u-PA) and monocyte chemoattractant protein 1

(MCP1) were reduced by 40–60% in late outgrowth endothelial progenitor-derived cells (LO-EPC) exposed to any flow environment, while MCP1, but not t-PA or u-PA, was decreased in HUVEC. **Conclusions:** Flow, in particular bidirectional, modifies the hemostatic balance in LO-EPC with increased TF and decreased plasminogen activator expression.

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Introduction

Atherosclerosis is the leading cause of morbidity and mortality in developed countries. Presentation of the disease may be in the form of myocardial infarction, stroke or peripheral arterial disease (PAD). The prevalence of PAD continues to increase and reaches about 30% in >65-year-old populations [1]. Interest in the management of PAD has increased, partly due to the technological advancement of devices and procedures used for the treatment. In fact, besides pharmacological management, treatment of PAD includes bypass surgery, endovascular techniques (with or without stent placement) or a combination of these in order to restore proper vascularization

to the ischemic limb. However, early or late bypass failure or intrastent stenosis are yet unsolved problems in the contemporary treatment of PAD [2]. Bypass surgery is performed whenever feasible using autologous veins to guarantee the highest patency rate possible. However, in about 30% of patients, autologous veins are not suitable for bypass surgery due to pre-existing venous diseases [3]. Moreover, in a substantial percentage of patients, venous bypass stenosis or occlusion occurs. This is mainly due to intimal hyperplasia and may lead to bypass angioplasty and/or reintervention. To obviate the lack of suitable autologous veins, synthetic material grafting has been developed. Nevertheless, the 5-year patency rate is less than 60% in above-knee prosthetic bypasses and even lower for below-knee reconstructions [4]. This may be due partly to the absence of an endothelial lining in synthetic grafts and partly to a compliance mismatch between graft and native artery [5, 6]. Coating of prosthetic inner surfaces with heparin or polyethylene oxide has been experimented without success [7, 8]. Endothelial cell (EC) seeding has also been studied, but only 1–14% of EC remain adherent on the prosthesis under flow conditions [9]. Tissue-engineering techniques have been developed in animal models to create vascular grafts [10–12]. Biodegradable polymer scaffolds or exogenous materials were seeded with vascular smooth muscle cells and EC cultured after blood vessel biopsy to create vascular grafts. Nevertheless, thrombosis occurs shortly after implantation.

Recent findings suggest that circulating endothelial progenitor-derived cells (EPC) are vitally important for the repair of endothelial damage. EPC are bone marrow-derived cells that are present in very low amounts in the circulation. They have the ability to migrate into ischemic tissues, contribute to the generation of new blood vessels and play a key role in vessel wall repair [13, 14]. EPC have been expanded *ex vivo* and a number of preclinical and clinical studies have shown their therapeutic potential. Thus, autologous EPC may be used to stimulate new blood vessel growth in ischemic tissues, to accelerate re-endothelialization of venous grafts, endothelialization of stents, for *ex vivo* seeding of arterial bypass grafts or for the *ex vivo* generation of artificial vascular grafts [15–18].

EPC have been widely studied and characterized *in vitro* under static culture conditions. However, *in vivo*, the vascular endothelium is continuously exposed to varying conditions of flow and shear stress. Normally, EC actively respond to this varying hemodynamic environment by changing cell morphology and gene expression. For example, it is well known that arterial blood flow at

branch points, bifurcations and curved regions is bidirectional (characterized by separations, cyclic flow reversals and a wall shear stress which is highly fluctuating but with an overall low mean value). Such a flow pattern contrasts with the unidirectional one found in nonbranching arterial segments and is characterized by absence or non-significant flow reversals and a steady wall shear stress. Interestingly, atherosclerotic lesions preferentially develop in areas of bidirectional flow while areas with unidirectional shear stress are relatively plaque free. We have shown that exposure of EaHy 926 cells, an endothelial-like cell line, to bidirectional shear stress increases expression of tissue factor (TF), an atherosclerosis-related gene [19].

The potential use of late outgrowth (LO)-EPC for therapeutics involving medium or large vessels makes it of paramount importance to study their behavior under conditions of shear and tensile stress normally found in human vessels. Therefore, the aim of our work was to study the expression by LO-EPC of coagulation/fibrinolysis proteins and inflammation-associated proteins after exposure to static conditions and different hemodynamic environments: low and high unidirectional shear stress and bidirectional shear stress.

Materials and Methods

Cell Isolation and Culture

Human umbilical cords and umbilical cord blood samples were collected from donors in accordance with Swiss and French legislation with written informed consent from the parents and approval by the local ethics committees. Blood was collected in a sterile tube containing heparin. LO-EPC were isolated and characterized as previously described [20, 21]. Briefly, human umbilical cord blood samples (30–50 ml) were collected in a sterile tube containing heparin. Mononuclear cells (MNC) were isolated, after 1:1 dilution in phosphate-buffered saline containing 2 mM EDTA, by density gradient centrifugation on a lymphocyte separation medium (Eurobio). After a 30-min centrifugation at 400 g, the MNC were washed 3 times in PBS/EDTA and preplated in RPMI/10% fetal calf serum for 24 h in plastic flasks. Nonadherent cells were plated onto 0.2% gelatin-coated 24-well plates (10^5 cells per well) and maintained in endothelial basal medium-2 supplemented with EGM-2 SingleQuots (EGM-2 medium; Clonetics) composed of a mixture of human VEGF, epidermal growth factor, fibroblast growth factor- β , insulin-like growth factor-1, hydrocortisone, ascorbic acid and heparin. The medium was changed every 4 days. The appearance of well-circumscribed colonies with a cobblestone morphology was monitored daily. For expansion, colonies were trypsinized and cells replated on a 35-mm plate (passage 1). Subsequently, confluent cells were trypsinized and replated in T75 flasks for further passages. Cells were used at passages 4–6. Culture of EPC from cord blood-derived nonadherent MNC, under these conditions, resulted in the emergence of ad-

herent colonies within 2–3 weeks. Cells rapidly expanded into a confluent monolayer displaying a cobblestone morphology and a homogeneous endothelial phenotype as evidenced by the uniform membrane expression of various endothelial antigens such as PECAM-1 (CD31), S-Endo1 (CD146), CD34, VE-cadherin (CD144), von Willebrand factor and KDR, whereas cells expressed neither leukocyte markers (CD45, CD14) nor the immaturity marker CD133. The cells took up Dil-ac-LDL and showed *Ulex europaeus* lectin binding. Taken together, the EPC morphology, time of appearance in culture, proliferation potential and phenotype are consistent with the features of LO-EPC previously described [20–23].

Umbilical vein-derived mature endothelial cells (HUVEC) were obtained by collagenase digestion of human umbilical cord veins and grown in EGM-2 medium in tissue culture dishes coated with 0.1% gelatin. Cells were used during passage 2.

Both EPC and HUVEC had a high proliferation potential and could easily be expanded to more than 10 billion cells in vitro (fig. 1).

Flow Experiments

Cord blood-derived LO-EPC and HUVEC were tested in an in vitro perfusion system described previously [19]. Briefly, 4 compliant transparent Sylgard tubes, 10 cm long and 6 mm in diameter at rest, were coated first with fibronectin and subsequently with LO-EPC or HUVEC and mounted in parallel on specially designed fittings (for a description see figure 1 of Mazzolai et al. [19]). Medium was perfused through the tubes from a reservoir continuously gassed by a 5% CO₂/95% air mixture to keep the pH constant. The laminar unidirectional flow was driven by a gear pump (Ismatech) controlled by a pulse generator (Hewlett-Packard) producing a 1-Hz sinusoidal flow rate. The bidirectional flow pattern was obtained by adding to the described system a second pump generating a cyclic flow reversal to reduce the mean flow rate generated by the first pump to almost zero. The temperature of the whole system was kept constant at 37°C and the viscosity of the flowing medium was kept constant ($1.07 \times 10^{-3} \text{ N m}^{-2} \text{ s}^{-1}$) by adding 2% dextran (Sigma-Aldrich) to the cell culture medium (DMEM; Gibco Invitrogen). Flow rates were measured using an ultrasonic flow meter (Transonic System Inc.) with a precision of 2%. Shear stress was calculated from viscosity, mean flow rate, and tube diameter measurements using a Womersley approximation [24]. Pressure was measured by pressure probes (Millar Instruments) inserted in each tube and kept in all experiments at $100 \pm 30 \text{ mm Hg}$. The Reynold's number (a dimensionless quantity which defines the point at which flow changes from laminar to turbulent) has been calculated to be $<2,000$ for both the bidirectional and the unidirectional flow conditions, and therefore, these flows must be considered laminar. A rigid shell was mounted around the tubes to avoid potential confounding effects of stretch.

Cell Staining

At the end of the flow experiments, tubes were longitudinally opened, the cell layer stained with crystal violet (0.25%) and analyzed under the microscope.

Surface TF Activity Assay

TF activity on the LO-EPC surface was assessed by measuring enzymatic factor X activation by the TF/FVIIa complex as previ-

Fig. 1. Proliferation potential of EPC compared to HUVEC obtained from the same donor. EPC and HUVEC were continuously passaged for a period of up to 70 days and total cell number determined. EPC are represented by closed symbols and HUVEC by open symbols. Each donor is identified by a particular symbol: triangles or circles.

ously described [19]. Briefly, each tube containing a confluent LO-EPC monolayer exposed for 24 h to bidirectional, unidirectional or static flow conditions was first rapidly rinsed with PBS to remove any detached cells. Subsequently, 2 ml buffer (Tris-HCl 50 mM, pH 8.3, NaCl 150 mM) containing 5 mM Ca⁺⁺, 0.5 µg/ml recombinant FVIIa and 10 µg/ml FX (Enzyme Research Laboratories) were added to each tube and left to incubate with the cells in a rotating support at 37°C. After 30-min incubation, aliquots were taken from each tube and a chromogenic substrate (chromozyme X; Roche) was added. At the end of the incubation time absorbance was measured at 410 nm. The LO-EPC from each tube were subsequently detached and processed for total protein determination using the Bio-RAD protein assay following the manufacturer's guidelines (Bio-Rad Laboratories GmbH). FXa formation rate was calculated from a standard curve established by using known amounts of FXa (Roche) run in parallel with the experimental samples. Results were measured as units of FXa activity per milligram of total cellular protein and expressed as fold change with respect to the corresponding static controls.

Tissue-Type Plasminogen Activator Antigen Measurements

Tissue-type plasminogen activator (t-PA) was measured by ELISA as previously described [24] with some modifications. In short: the 96-well plates were coated by adding 100 µl/well of monoclonal anti-t-PA antibodies ESP1 and ESP5 (American Diagnostica) at 1 µg/ml each in carbonate buffer (50 mM NaCO₃, pH 9.6). After 3 washes with TBS-Tween (20 mM Tris, 140 mM NaCl, 0.1% Tween-20 pH 7.4) the plates were blocked with TBS-Tween (3% bovine serum albumin) for 2 h at 37°C. Samples and standards were diluted in blocking solution (0.1 ml/well) and then

Table 1. Primer sequences (5' to 3') used for determination of mRNA levels of the indicated proteins by QRT-PCR

Protein	Forward	Reverse
GAPDH	GGTGAAGGTCGGAGTCAAC	CCATGGGTGGAATCATATTG
E-selectin	ATCATCCTGCAACTTCACC	ACACCTCACCAAACCCCTC
VCAM-1	CATGGAATTGCAACCCAAACA	GACCAAGACGGTTGTATCTCTGG
Tissue factor	CTACTGTTTCAGTGTTCAAGCAGTGA	CAGTGCAATATAGCATTTCAGTAGC
MCP-1	GTCACCTGCTGCTATAACTTC	TGCTGCTGGTGATTCTCTA
IL-6	GACCCAACCACAAATGCCA	GTGATGTCCTGCAGCCACTG
PAI 1	AAGGGTCTGCTGTGCACCAT	AAACACCCTCACCCCGAAGT
tPA	CCGGCTACGGCAAGCA	AGCGGCTGGATGGGTACA
uPA	TCAAAAACCTGCTATGAGGGGA	GGGCATGGTACGTTTGCTG
uPAR	TATTCCTCGAAGCCGTACCTC	GGTGGCGGTCATCCTTTGG
KLF2	CCAACTGCGGCAAGACCTAC	AGTCGACCCAGGCTACATGTG

added and incubated for 2 h at 37°C. After 3 washes, 100 µl of biotinylated sheep anti-human t-PA antibody (diluted 1:1,000; Abcam) was added and incubated for 2 h at 37°C. After 3 washes and addition of 100 µl of streptavidin-HRP 1:200 (R&D Systems), plates were incubated for 20 min at 37°C. The plates were washed and 100 µl of substrate solution (R&D Systems) was added to each well and incubated at room temperature in the dark. The reaction was stopped with 50 µl 2 N H₂SO₄ and the optical density determined at 450 nm. Antigen concentrations in cell extracts are expressed as nanogram t-PA per milligram of total protein in cell extracts as measured by the BCA protein assay (Pierce).

RNA Extraction and Quantitative Reverse Transcriptase Real-Time PCR

Total RNA was extracted from cells and treated with DNaseI using the RNeasy® Micro kit (Qiagen). Five microliters of total RNA was used for reverse transcription using the ImPromII™ reverse transcription system and Oligo-d(T15) primers (Promega). Negative controls were processed in parallel without reverse transcriptase. Relative mRNA levels of a panel of marker proteins characteristic of EC or proteins important for angiogenesis were quantified by quantitative reverse transcriptase real-time PCR (QRT-PCR) using cDNA obtained from the reverse transcription reactions as template, with an Applied Biosystems StepOne instrument and SYBR-Green master mix reagent (Applied Biosystems). The total volume of the reaction was 10 µl. Data were analyzed using the $\Delta\Delta C_T$ method and Applied Biosystems Prism software, according to the manufacturer's instructions. Human GAPDH expression was used as the housekeeping gene control. Individual samples were subjected to duplicate or triplicate QRT-PCR reactions and ΔC_T values calculated. The primer sequences are given in table 1.

Statistical Analysis

Results are expressed as means $\pm$ SEM. Statistical analysis was performed using ANOVA and the nonparametric Kruskal-Wallis test. If necessary a posttests analysis (Dunn's test) was performed. Results with a $p < 0.05$ were considered to be statistically significant.

Results

LO-EPC Morphology after Exposure to Various Flow Conditions

LO-EPC exposed for 24 h to unidirectional (shear stress values of either 0.3 ± 0.1 or 6 ± 3 dynes/cm²) or bidirectional (0.3 ± 3 dynes/cm²) shear stress did not detach and remained confluent throughout the experimental period. Cells exposed to unidirectional shear stress elongated significantly, compared with unstressed controls, and oriented along the direction of flow (fig. 2). On the contrary, cells exposed to bidirectional shear stress showed a random distribution (fig. 2). This morphological behavior has been observed before with mature, human umbilical vein-derived EC [26].

TF Activity and mRNA Levels Are Increased in LO-EPC Exposed to Pulsatile Bidirectional Shear Stress

Previously, we observed that EaHy 926 mature EC show increased surface TF activity when subjected to bidirectional, but not unidirectional flow [19]. To investigate whether LO-EPC would behave similarly, cells were exposed to various hemodynamic conditions and surface TF activity was measured. LO-EPC exposed to bidirectional shear stress showed a 2.5 ± 0.3 -fold increase in TF activity ($n = 4$; $p < 0.05$), whereas for HUVEC a nonsignificant 6.6 ± 5.9 -fold increase was observed ($n = 4$). No significant changes in TF activity of LO-EPC or HUVEC were observed after exposure to high or low unidirectional shear stresses. TF mRNA expression determined by quantitative RT-PCR showed a significant increase after bidirectional shear exposure. This increase was also significant following unidirectional low shear stress (ta-

Fig. 2. Morphology of EPC cultured on fibronectin-coated Sylgard tubes under static conditions, unidirectional flow (0.3 ± 0.1 dynes/cm²), or bidirectional flow (0.3 ± 3 dynes/cm²). Note the random disposition of EPC under conditions of unidirectional flow.

ble 2). All flow conditions induced an increase in TF mRNA in HUVEC.

Gene Expression Analysis in LO-EPC Exposed to Various Flow Conditions

We also investigated by quantitative RT-PCR analysis the effect of unidirectional and bidirectional flows on the expression by LO-EPC and HUVEC of several fibrinolytic (t-PA, urokinase-type plasminogen activator, u-PA, u-PA receptor, u-PAR, and PAI-1) and inflammation-associated proteins (E-selectin, VCAM-1, monocyte chemoattractant protein 1, MCP-1, and IL-6). As a positive control for flow-induced changes we measured mRNA levels of Krüppel-like factor 2 (KLF2). Expression levels in cells exposed to either unidirectional or bidirectional flow were compared to static cultures. For bidirectional and unidirectional flow conditions, a reduction in MCP-1, t-PA, u-PA and u-PAR mRNA was observed in LO-EPC (table 2). In HUVEC, a flow-mediated reduction in MCP1 was observed for all flow conditions and for u-PAR only at high shear stress unidirectional flow. At a high shear stress we observed an increase in t-PA mRNA and a reduction in u-PA mRNA that did not reach significance. In EPC, bidirectional flow conditions reduced cell-associated t-PA antigen from 2.25 ± 0.4 to 1.15 ± 0.15 ng/mg ($\pm$ SEM; $n = 6$; $p < 0.05$). In HUVEC, bidirectional flow conditions reduced cell-associated t-PA antigen from 1.26 ± 0.25 to 0.47 ± 0.05 ng/mg ($\pm$ SEM; $n = 4$; $p <$

0.05). No significant changes in t-PA antigen were observed in EPC or HUVEC under low or high shear stress unidirectional flow (with differences between flow and static conditions of less than 30%). PAI-1 was increased under all conditions, but differences were only significant with low shear stress unidirectional flow. The changes observed for E-selectin, VCAM, IL6 and PAI1 gene expression in LO-EPC and HUVEC were not significant (table 2). KLF2 expression was significantly increased in LO-EPC and HUVEC only at conditions of high shear stress unidirectional flow.

Discussion

The results presented herein show the expression of various proteins relevant for coagulation/fibrinolysis or for inflammation by LO-EPC exposed to various flow environments mimicking those found in human medium and large vessels. Our results provide important information for the further development of these cells for human cell therapy. We observed, as compared to static LO-EPC, a flow-mediated increase in TF, which was highest under conditions of bidirectional flow and lowest under conditions of unidirectional flow. The changes in TF expression are comparable to that observed for HUVEC (this report) and our previous results [19] using the same flow system and EaHy 926 cells, a permanent EC-like line de-

Table 2. mRNA analysis by QRT-PCR of the effect of bidirectional and unidirectional flow on the expression of hemostasis and inflammation proteins by EPC and HUVEC

			Static	Bidirectional 0.3 ± 3 dynes/cm ²	Unidirectional 0.3 ± 0.1 dynes/cm ²	Unidirectional 6 ± 3 dynes/ cm ²
Tissue factor	EPC	1		7.0 ± 0.6*	4.9 ± 0.5*	2.2 ± 0.3
	HUVEC	1		8.9 ± 2.3*	12.8 ± 1.8*	7.3 ± 1.2*
E-selectin	EPC	1		2.2 ± 0.5	8.9 ± 4	2.2 ± 1.4
	HUVEC	1		3.5 ± 0.7*	2.1 ± 1.1	1.3 ± 0.3
MCP1	EPC	1		0.7 ± 0.2*	0.6 ± 0.2	0.4 ± 0.2*
	HUVEC	1		0.5 ± 0.07*	0.4 ± 0.08*	0.6 ± 0.3*
VCAM1	EPC	1		2.2 ± 0.7	1.6 ± 0.7	0.3 ± 0.1
	HUVEC	1		0.6 ± 0.2	0.8 ± 0.2	1.0 ± 0.5
IL-6	EPC	1		1.4 ± 0.2	1.1 ± 0.2	0.8 ± 0.2
	HUVEC	1		1.5 ± 0.1	1.1 ± 0.4	0.7 ± 0.4
PAI -1	EPC	1		1.5 ± 0.2	1.3 ± 0.3	1.0 ± 0.1
	HUVEC	1		1.7 ± 0.3	1.7 ± 0.2*	1.8 ± 0.4
tPA	EPC	1		0.4 ± 0.1*	0.5 ± 0.1	0.4 ± 0.1*
	HUVEC	1		1.0 ± 0.3	1.3 ± 0.1	1.8 ± 0.3*
uPA	EPC	1		0.5 ± 0.1*	0.6 ± 0.1	0.5 ± 0.1*
	HUVEC	1		1.0 ± 0.2	0.8 ± 0.3	0.4 ± 0.3
uPAR	EPC	1		0.5 ± 0.03*	0.8 ± 0.1	0.8 ± 0.2
	HUVEC	1		0.9 ± 0.05	0.7 ± 0.1	0.6 ± 0.1*
KLF2	EPC	1		1.1 ± 0.08	1.1 ± 0.1	3.1 ± 0.7*
	HUVEC	1		0.8 ± 0.06*	1.1 ± 0.2	4.7 ± 0.1*

Results are expressed as fold change compared to cells cultured under static conditions. n = 6–15 in each EPC group and 4–6 in each HUVEC group. * p < 0.05 versus static.

rived by fusion of umbilical vein EC and A549 human lung carcinoma cells [27]. We observed in LO-EPC, but not in HUVEC, a 2-fold reduction in expression of the fibrinolytic proteins t-PA and u-PA. For these proteins no differences were observed between unidirectional or bidirectional flow. The combination of increased expression of TF and reduced expression of t-PA and u-PA suggests that flow induces a moderate shift in LO-EPC towards a more procoagulant phenotype, with bidirectional conditions having the greatest effect. The question then may be raised as to the utility of using EPC for revascularization therapy when they have an increased procoagulant phenotype under flow conditions. It has to be stressed, however, that an exposed subendothelial matrix is highly thrombogenic and it is to be expected that coat-

ing with EC, even expressing an increase in TF under flow conditions, may have beneficial effects. For our studies we used a previously described in vitro flow system, designed to closely resemble flow conditions encountered in vivo in medium and large vessels [19]. It has to be stressed that this in vitro flow model system cannot precisely reproduce important pathophysiological conditions, such as arterial stenoses or branch points, nor can it be used to study flow effects on endothelial surface blood cell interactions. The major characteristics of the used flow system are: (1) the LO-EPC or HUVEC were grown on a tubing, Sylgard, that has a compliance similar to that of human arteries, which also takes into account the effect of vascular stretch; (2) unidirectional or bidirectional flow conditions were sinusoidal with a frequency (1 Hz) and shear stresses normally encountered in vivo; (3) cells were grown on fibronectin, a matrix component typically encountered in regions of disturbed flow. The latter is important because inflammation or vascular injury may lead to changes in the composition of subendothelial extracellular matrix (ECM) from collagen IV and laminin to proteins characteristic of wounds and inflammation such as fibronectin and fibrinogen [28, 29]. Changes in ECM composition lead to a shift of integrin expression mediating EC binding. Whereas collagen IV binds integrins $\alpha 2\beta 1$ and $\alpha 1\beta 1$, fibronectin interacts with integrins $\alpha 6\beta 1$ and $\alpha 6\beta 4$ [30, 31]. Shear stress-mediated activation of the inflammatory transcription factor NF- κ B critically depends on ECM composition. Thus, in EC grown on collagen, shear stress leads to decreased NF- κ B nuclear translocation and decreased surface expression of leukocyte adhesion molecule ICAM-1. On the contrary, in EC grown on fibronectin or fibrinogen, shear stress increases NF- κ B translocation and ICAM-1 expression [28]. The lack of shear stress responses in EC cultured on collagen IV is mediated by p38 activation induced by collagen-integrin $\alpha 2\beta 1$ interactions [31].

In the present study we used so-called late-outgrowth EPC. These cells are derived from circulating progenitor cells that are present in blood in extremely low numbers (for example, approximately 8 LO-EPC colonies can be derived from 20 ml of cord blood and less than 1 colony per 20 ml of adult blood [32]), but can be expanded to very large cell numbers in vitro (fig. 1). The LO-EPC were obtained after more than 4 weeks outgrowth in culture, exhibited the cobblestone morphology that is typical for mature EC [32, 33] and expressed endothelial marker proteins and were negative for leukocyte marker proteins. LO-EPC, in contrast to EC derived from adult blood ves-

sels, have a high proliferation potential, which justifies their designation as endothelial progenitor cells. The high proliferation potential of LO-EPC distinguishes these cells from the so-called early-outgrowth EPC, also named circulating angiogenic cells, which facilitate blood vessel formation by releasing angiogenic growth factors and proteases, but cannot be propagated further in vitro; LO-EPC are also distinct from circulating EC, which are the result of vascular damage and do not proliferate significantly [for a discussion, see 34]. In the context of re-endothelialization of vein grafts or of arteries after angioplasty or stenting, the use of LO-EPC appears to be more appropriate, because of their ability to directly coat the vascular grafts. The recent description of a more efficient way to isolate LO-EPC with high proliferation potential from adult blood opens the perspective of using these cells in an autologous manner [35].

A concept implying the effect of flow on endothelial gene expression is the inflammatory activation of EC in regions of disturbed flow close to branch points. Under our experimental conditions, LO-EPC subjected to unidirectional low shear or bidirectional flow exhibited a small, but not significant, increase in expression of the leukocyte adhesion molecules VCAM-1 and E-selectin, but a decrease in the chemoattractant protein MCP-1. No significant changes in IL-6 and PAI-1 were observed. Our data, therefore, do not support an increased expression of inflammation marker proteins as an important factor distinguishing bidirectional (oscillatory) flow from unidirectional flow. A consistent finding was the increase in TF activity and mRNA expression by LO-EPC with highest levels under conditions of bidirectional flow, intermediate levels with unidirectional flow at low or high shear rates and lowest levels when cells were cultured under static conditions. Similar flow-induced changes in TF were observed for HUVEC. Inflammation is one mechanism by which expression of TF by EC may be increased. Induction of TF expression by inflammatory mediators requires activation of a unique NF- κ B-like element, named TF- κ B, and of an AP-1/TRE-like element in the TF promoter [36]. As expression of MCP-1, which depends on NF- κ B- and AP-1/TRE-like elements, was decreased under flow conditions, we have to assume that other factors also play an important role. One factor may be that the effect of shear stress on MCP-1 expression is not constant, but transient, reaching a maximum at 4 h and returning to normal within 24 h [37]. Another factor may be a differential sensitivity of NF- κ B and TF- κ B to histone deacetylases [38]. Further work needs to be done to explain the opposing sensitivities of TF and MCP-1 to shear stress.

We investigated the expression of KLF2 in EPC and EC under the various flow conditions. KLF2 is a transcription regulator that is highly expressed in EC that has anti-inflammatory activities and plays an important role in vascular development [39]. Under our flow conditions KLF2 was only increased under conditions of high shear unidirectional flow but not of low shear flow or bidirectional flow. Our findings are in agreement with previous findings of a strong increase in KLF2 expression in cultured human and murine endothelial cell under high shear stress unidirectional flow [40, 41] and with in vivo findings of a low KLF2 expression at arterial bifurcations and high expression at sites of high shear stress [42].

We observed a significant decrease in t-PA mRNA expression in LO-EPC, but not in HUVEC, under bidirectional or unidirectional flow. In contrast to our findings, Yang et al. [43] observed in EPC grown on fibronectin a significant increase in t-PA expression, albeit only at higher shear stress values than used in the present study (≥ 15 vs. 6 ± 0.3 dynes/cm²). A critical difference between our study and that of Yang et al. [43] is the cell type used. The results presented in the present report derive from late-outgrowth EPC, whereas the results of Yang et al. [43] were obtained with early-outgrowth EPC. The latter are not clonally related the late-outgrowth EPC used in the present study [44]. Furthermore, early- and late-outgrowth EPC differ in cell morphology and expression of endothelial marker genes [22]. The expression by early-outgrowth EPC of the leukocyte marker proteins CD14 and CD45 suggests that they represent a subpopulation of monocytes that stimulate the formation of new blood cells by secreting angiogenic growth factors. In contrast to late-outgrowth EPC, the early-outgrowth EPC are not incorporated in new blood vessels in vivo [45, 46]. Several studies addressed the effect of shear stress on the expression of t-PA by cultured mature human EC. In one study a 2-fold increase in t-PA release was observed in human umbilical vein-derived EC grown on gelatin, but no information was given on the matrix on which cells were grown [47]. In another study, a small but not significant increase in t-PA mRNA was observed [48]. In the present study, a high shear stress unidirectional flow, but not low shear stress or bidirectional flow, induced a small increase in t-PA mRNA in HUVEC, whereas bidirectional shear stress induced a significant reduction. Our finding of a significantly increased expression of t-PA by HUVEC under conditions of high shear stress is in agreement with these previous results. Further work needs to be done to determine the mechanism underlying the significant decrease in t-PA expression under high shear stress flow

conditions in LO-EPC as compared to an increase in HUVEC.

A clear understanding of the effect of flow on the behavior of EPC is of critical importance for their therapeutic use. The results presented here provide additional information in this respect and suggest that the effects of flow on hemostatic and inflammatory gene expression by EC or EPC is dependent on whether flow is unidirectional or bidirectional and on the magnitude of shear stress.

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References

- Diehm C, Schuster A, Allenberg JR, Darius H, Haberl R, Lange S, Pittrow D, von Stritzky B, Tepohl G, Trampisch HJ: High prevalence of peripheral arterial disease and co-morbidity in 6,880 primary care patients: cross-sectional study. *Atherosclerosis* 2004;172:95–105.
- Owens CD, Ho KJ, Conte MS: Risk factors for failure of lower-extremity revascularization procedures: are they different for bypass and percutaneous procedures? *Semin Vasc Surg* 2008;21:143–153.
- Mythie H, Dahl T, Lundbom J, Saether O, Gronningsaether A: Imaging of the superficial veins for suitability of bypass surgery; in Greenhalgh RM (ed): *Vascular Imaging for Surgeons*. London, WB Saunders Company, 1995, pp 353–360.
- Klinkert P, Schepers A, Burger DH, van Bockel JH, Breslau PJ: Vein versus polytetrafluoroethylene in above-knee femoropopliteal bypass grafting: five-year results of a randomized controlled trial. *J Vasc Surg* 2003;37:149–155.
- Berger K, Sauvage L, Rao A, Wood S: Healing of arterial prosthesis in man: its incompleteness. *Ann Surg* 1972;175:118–127.
- Herring M, Smith J, Dalsing M: Endothelial seeding of polytetrafluoroethylene femoral popliteal bypasses: the failure of low-density seeding to improve patency. *J Vasc Surg* 1994;20:650–655.
- Devine A, Hons B, Mc Collum C: Heparin-bonded Dacron or polytetrafluoroethylene for femoropopliteal bypass grafting: a multicenter trial. *J Vasc Surg* 2001;33:533–539.
- Kidane A, Lantz GC, Jo S: Surface modification with PEO-containing triblock copolymer for improved biocompatibility: in vitro and ex vivo studies. *J Biomater Sci Polym Ed* 1999;10:1089–1105.
- Rosenman JE, Kempczinsky RF, Pearce WH, Silberstein EB: Kinetics of endothelial cell seeding. *J Vasc Surg* 1985;2:778–784.
- Weinberg CB, Bell E: A blood vessel model constructed from collagen and cultured vascular cells. *Science* 1986;231:397–400.
- L'Heureux N, Paquet S, Labbe R: A completely biological tissue-engineered human blood vessel. *FASEB J* 1998;12:47–56.
- Niklason LE, Gao J, Abbott WM: Functional arteries grown in vitro. *Science* 1999;284:489–493.
- Rafii S, Lyden D: Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med* 2003;9:702–712.
- Kawamoto A, Asahara T: Role of progenitor endothelial cells in cardiovascular disease and upcoming therapies. *Catheter Cardiovasc Interv* 2007;70:477–484.
- Dimmeler S, Zeiher AM: Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? *J Mol Med* 2004;82:671–677.
- Griese DP, Ehsan A, Melo LG, Kong D, Zhang L, Mann MJ, Pratt RE, Mulligan RC, Dzau VJ: Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy. *Circulation* 2003;108:2710–2715.
- Moran AM, Schoen FJ, Atala A, Soker S, Bischoff J, Mayer JE Jr: Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. *Nat Med* 2001;7:1035–1040.
- Iwami Y, Masuda H, Asahara T: Endothelial progenitor cells: past, state of the art, and future. *J Cell Mol Med* 2004;8:488–497.
- Mazzolai L, Silacci P, Bouzourene K, Daniel F, Brunner H, Hayoz D: Tissue factor activity is upregulated in human endothelial cells exposed to oscillatory shear stress. *Thromb Haemost* 2002;87:1062–1068.
- Bompais H, Chagraoui J, Canon X, Crisan M, Liu XH, Anjo A, Tolla-Le Port C, Leboeuf M, Charbord P, Bikfalvi A, Uzan G: Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells. *Blood* 2004;103:2577–2584.
- Basire A, Sabatier F, Ravet S, Lamy E, Mialhe A, Zabouo G, Paul P, Gurewich V, Sampol J, Dignat-George F: High urokinase expression contributes to the angiogenic properties of endothelial cells derived from circulating progenitors. *Thromb Haemost* 2006;95:678–688.
- Liu JW, Pernod G, Dunoyer-Geindre S, Fish RJ, Yang H, Bounameaux H, Kruithof EK: Promoter dependence of transgene expression by lentivirus-transduced human blood-derived endothelial progenitor cells. *Stem Cells* 2006;24:199–208.
- Liu JW, Dunoyer-Geindre S, Serre-Beinier V, Mai G, Lambert JE, Fish RJ, Pernod G, Buehler L, Bounameaux H, Kruithof EK: Characterization of endothelial-like cells derived from human mesenchymal stem cells. *J Thromb Haemost* 2007;5:826–834.
- He X, Ku DN, Moore JE: Simple calculation of the velocity profiles for pulsatile flow in a blood vessel using Mathematica. *Ann Biomed Eng* 1993;21:45–49.
- Huber D, Cramer EM, Kaufmann JE, Meda P, Massé JM, Kruithof EK, Vischer UM: Tissue-type plasminogen activator (t-PA) is stored in Weibel-Palade bodies in human endothelial cells both in vitro and in vivo. *Blood*. 2002;99:3637–3645.
- Zhao S, Suciu A, Ziegler T, Moore JE Jr, Bürki E, Meister JJ, Brunner HR: Synergistic effects of fluid shear stress and cyclic circumferential stretch on vascular endothelial cell morphology and cytoskeleton. *Arterioscler Thromb Vasc Biol* 1995;15:1781–1786.
- Edgell CJ, McDonald CC, Graham JB: Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* 1983;80:3734–3737.
- Orr AW, Sanders JM, Bevard M, Coleman E, Sarembock IJ, Schwartz MA: The subendothelial extracellular matrix modulates NF- κ B activation by flow: a potential role in atherosclerosis. *J Cell Biol* 2005;169:191–202.
- Hahn C, Schwartz MA: The role of cellular adaptation to mechanical forces in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2008;28:2101–2107.

- 30 Belkin AM, Stepp MA: Integrins as receptors for laminins. *Microsc Res Tech* 2000;51:280–301.
- 31 Heino J: The collagen receptor integrins have distinct ligand recognition and signaling functions. *Matrix Biol* 2000;19:319–323.
- 32 Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC: Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood* 2004;104:2752–2760.
- 33 Lin Y, Weisdorf DJ, Solovey A, Hebbel RP: Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000;105:71–77.
- 34 Prater DN, Case J, Ingram DA, Yoder MC: Working hypothesis to redefine endothelial progenitor cells. *Leukemia* 2007;21:1141–1149.
- 35 Reinisch A, Hofmann NA, Obenauf AC, Kashofer K, Rohde E, Schallmoser K, Flicker K, Lanzer G, Linkesch W, Speicher MR, Strunk D: Humanized large-scale expanded endothelial colony-forming cells function in vitro and in vivo. *Blood*. 2009;113:6716–6725.
- 36 Parry GC, Mackman N: Transcriptional regulation of tissue factor expression in human endothelial cells. *Arterioscler Thromb Vasc Biol* 1995;15:612–621.
- 37 Shyy YJ, Hsieh HJ, Usami S, Chien S: Fluid shear stress induces a biphasic response of human monocyte chemotactic protein 1 gene expression in vascular endothelium. *Proc Natl Acad Sci USA* 1994;91:4678–4782.
- 38 Wang J, Mahmud SA, Bitterman PB, Huo Y, Slungaard A: Histone deacetylase inhibitors suppress TF- κ B-dependent agonist-driven tissue factor expression in endothelial cells and monocytes. *J Biol Chem* 2007;282:28408–28418.
- 39 Atkins GB, Jain MK: Role of Krüppel-like transcription factors in endothelial biology. *Circ Res* 2007;100:1686–1695.
- 40 Dekker RJ, van Soest S, Fontijn RD, Salamanca S, de Groot PG, VanBavel E, Pannekoek H, Horrevoets AJ: Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Krüppel-like factor (KLF2). *Blood* 2002;100:1689–1698.
- 41 Huddleson JP, Ahmad N, Lingrel JB: Up-regulation of the KLF2 transcription factor by fluid shear stress requires nucleolin. *J Biol Chem* 2006;281:15121–15128.
- 42 Dekker RJ, van Thienen JV, Rohlena J, de Jager SC, Elderkamp YW, Seppen J, de Vries CJ, Biessen EA, van Berkel TJ, Pannekoek H, Horrevoets AJ: Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes. *Am J Pathol* 2005;167:609–601.
- 43 Yang Z, Tao J, Wang JM, Tu C, Xu MG, Wang Y, Pan SR: Shear stress contributes to t-PA mRNA expression in human endothelial progenitor cells and nonthrombogenic potential of small diameter artificial vessels. *Biochem Biophys Res Commun* 2006;342:577–584.
- 44 Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA: Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principles. *Blood* 2007;109:1801–1809.
- 45 Rehman J, Li J, Orschell CM, March KL: Peripheral blood ‘endothelial progenitor cells’ are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation* 2003;107:1164–1169.
- 46 Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB: Characterization of two types of endothelial progenitor cells and their different contributions to neovasclogenesis. *Arterioscler Thromb Vasc Biol* 2004;24:288–293.
- 47 Diamond SL, Eskin SG, McIntire LV: Fluid flow stimulates tissue plasminogen activator secretion by cultured human endothelial cells. *Science* 1989;243:1483–1485.
- 48 McCormick SM, Eskin SG, McIntire LV, Teng CL, Lu CM, Russell CG, Chittur KK: DNA microarray reveals changes in gene expression of shear stressed human umbilical vein endothelial cells. *Proc Natl Acad Sci USA* 2001;98:8955–8960.
- 49 Fernandez P, Bourget C, Bareille R, Daculsi R, Bordenave L: Gene response in endothelial cells cultured on engineered surfaces is regulated by shear stress. *Tissue Eng* 2007;13:1607–1614.