High urokinase expression contributes to the angiogenic properties of endothelial cells derived from circulating progenitors

Agnès Basire1,2, Florence Sabatier1,2, Sophie Ravet3, Edouard Lamy1, Agnès Mialhe1, Gwladys Zabouo1, Pascale Paul1,2, Victor Gurewich3, José Sampol1, Françoise Dignat-George1,2
1INSERM U608, Laboratoire d’Hématologie et d’Immunologie, UFR de Pharmacie, Université de la Méditerranée, Marseille, France
2Laboratoire d’Hématologie, Centre Hospitalier Universitaire La Conception, Marseille, France
3Vascular Research Laboratory, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA

Summary
Endothelial progenitor cells (EPC) display a unique ability to repair vascular injury and promote neovascularization although the underlying molecular mechanisms remain poorly understood. Urokinase-type plasminogen activator (uPA) and its receptor (uPAR) play a critical role in cell migration and angiogenesis by facilitating proteolysis of extracellular matrix. The aim of the present study was to characterize the uPA/uPAR-dependent proteolytic potential of EPC outgrown from human umbilical cord blood and to analyze its contribution to their angiogenic properties in vitro. Cells derived from EPC (EPDC), presenting typical features of late outgrowth endothelial cells, were compared to mature endothelial cells, represented by human umbilical vein endothelial cells (HUVEC). Using quantitative flow cytometry, enzyme-linked immunosorbent assays and zymography, we demonstrated that EPDC displayed higher levels of uPA and uPAR. In conditioned culture media, uPA-dependant proteolytic activity was also found to be significantly increased in EPDC. This activity was paralleled by a higher secretion of pro-metalloproteinase-2 (pro-MMP-2). Inhibition of EPDC-associated uPA by monoclonal antibodies that block either uPA activity or receptor binding, significantly reduced proliferation, migration and capillary like tube formation. Moreover, tumor necrosis factor-alpha and vascular endothelial growth factor, known to be locally secreted in ischemic areas, further increased the proteolytic potential of EPDC by up-regulating uPA and uPAR expression respectively. The EPDC response to these factors was found to be more pronounced than that of HUVEC. In conclusion, these findings indicated that EPDC are characterized by high intrinsic uPA/uPAR-dependent proteolytic potential that could contribute to their invasive and angiogenic behaviour.

Keywords
Endothelial progenitor, urokinase, angiogenesis, proteolysis

Introduction
The discovery of endothelial progenitor cells (EPC) in adult peripheral blood (1) has challenged the traditional understanding of post-natal angiogenesis by demonstrating that bone-marrow-derived immature circulating cells can take part in endothelial regeneration and neovascularization, a process termed "vasculogenesis" (2, 3). Impaired vasculogenesis, due to depletion or dysfunction of circulating EPC, associated to cardiovascular risk factors, significantly compromises vascular repair and contributes to the pathogenesis of vascular diseases (4, 5). These observations have rapidly translated into new pro-angiogenic therapeutic strategies using transplantation of stem cell fractions containing EPC or in vitro expanded EPC to promote neovascularization after ischemic events (6, 7). Despite encouraging results, the mechanisms underlying the specific ability of EPC to contribute to the elongation of growing vascular network remain poorly understood (8). In vitro studies, based on the capacity of EPC to differentiate in colonies with endothelial phenotype, have highlighted functional profiles that could be critically involved in their angiogenic properties. EPC-derived cells secrete large amounts of angiogenic factors (vascular endothelial growth factor, VEGF) or cytokines (Interleukine-8) which stimulate sprouting of resident mature endothelial cells in a paracrine manner (9, 10). In addition, EPC have inherent proliferative properties and an unusual resistance to various stresses associated to the ischemic environment (11). More recently Urbich, et al. reported that a high expression of the protease cathepsin-L in EPC is essential for their invasive activity and their capacity to integrate into ischemic tissues (12).
During angiogenesis, proteolytic enzymes permit endothelial cells to migrate through the basement membrane and into the stroma, enabling their organization into tubular structures (13). Endothelial-associated proteolytic activity is related to urokinase-type plasminogen activator (uPA), a serine protease secreted as a pro-enzyme (pro-uPA) which is activated by plasmin, kallikrein or thermolysin (14). UPA binds with high affinity to a specific cell-surface receptor (uPAR) which helps to target its plasminogen-activating activity. Cell-surface generation of plasmin provides a focal and directional proteolysis through both direct degradation of extracellular matrix components (type IV collagen, fibronectin and laminin) and catalytic activation of metalloproteinases (MMP). Animal models have firmly established that serine proteases of the plasminogen activator/plasmin system are required for tumor or ischemia induced angiogenesis (15, 16). UPA deficient mice presented impaired post-infarction myocardial revascularization related to a reduced invasive capacity of endothelial cells (17). Despite strong evidence for the involvement of uPA in post-natal neovascularization, to our knowledge the expression of this proteolytic pathway by EPC is still unknown.

The aim of the present study was to analyze the uPA/uPAR-dependent proteolytic potential of EPC-derived cells (EPDC) from human umbilical cord blood, compared to human umbilical vein endothelial cells (HUVEC), and to evaluate its contribution to their angiogenic properties in vitro. In addition, we studied whether factors known to be secreted in ischemic tissues such as VEGF, basic fibroblast growth factor (bFGF) and tumor necrosis factor-alpha (TNF-α), affect the expression of uPA and uPAR by EPDC.

**Materials and methods**

**Isolation and culture of EPC from cord blood and culture of HUVEC**

Human umbilical cord blood samples (30–50 ml) from donors were collected, in compliance with French legislation, in a sterile tube containing heparin. Mononuclear cells (MNC) were isolated by density gradient centrifugation. Briefly, blood was diluted 1:1 in phosphate-buffered saline containing 2 mM ethylenediaminetetraacetic (PBS/EDTA) and layered over lymphocyte separation medium (Eurobio, Les Ulis, France). After a 30 min centrifugation at 400 g, MNC were washed three times in PBS/EDTA and pre-plated in RPMI/10% fetal calf serum (FCS) for 24 h in plastic flasks to remove mononuclear cells. Non-adherent cells were plated onto 0.2% gelatin-coated 24-well plates (10^4 cells per well) and maintained in endothelial basal medium-2 (EBM-2) supplemented with EGM-2 SingleQuots (EGM-2 medium, Clonetics, Walkersville, MD, USA). The medium was changed every 4 days. The appearance of well-circumscribed colonies with a cobblestone morphology was monitored daily. For expansion of EPDC, colonies were trypsinized and cells were replated on a 35 mm plate (passage 1). Subsequently, confluent cells were trypsinized and re-plated in T75 flasks for further passages. Cells at passages 2, 4 and 6 were used (P2, P4 and P6). HUVEC were isolated according to the method of Jaffé, et al. (18), seeded on 0.2% gelatin-coated plates and grown in EGM-2 medium. HUVEC were used at passages 1 to 3. All the cells were maintained under standard conditions (humidified atmosphere, 5% CO₂, 37°C).

For stimulation experiments, cells were maintained for 24 hours in EBM-2 supplemented with SingleQuots omitting growth factors (VEGF, IGF, hFGF, hEGF), EGM-2 wGF, and then stimulated for 3, 6, 12 and 24 h with 5, 10, 20, 50 or 100 ng/ml of human recombinant TNF-α (Tebu-Bio, Le Perray en Yvelines, France, specific activity > 2.10⁷ units/mg) or bFGF or VEGF (R&D systems, Minneapolis, MN, USA).

To determine whether the highest dose of TNF-α would cause cytotoxicity, the LDH release, an indicator of loss of plasma membrane integrity, was measured using a LDH kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions. HUVEC and EPDC were also stained with Annexin V-FITC and Propidium iodide (Annexin V-FITC kit, Beckman Coulter Immunotech, Marseille, France) and analyzed by flow cytometry to detect apoptosis and necrosis.

**Immunocytochemistry analysis**

The colonies were tested for the uptake of Dil acetylated low-density lipoprotein (Dil-ac-LDL, Molecular Probes, Eugene, OR, USA) and the binding of FITC-conjugated lectin from Ulex europaeus (Sigma, St Quentin Fallavier, France). The expression of endothelial specific antigens was also tested after indirect immunostaining using primary antibodies directed against vWF (Dako, Trappes, France) and VE-Cadherin (CD144, clone TEA1/31, Beckman Coulter Immunotech) and analyzed by fluorescence microscopy as previously described (19).

**Quantitative flow-cytometry analysis**

Confluent EPDC and HUVEC were trypsinized and labeled for 45 min at 4°C with monoclonal antibodies directed against human uPA (clone 3471, IgG1, American Diagnostica, Greenwich, CT, USA), human uPAR (clone 3936, IgG2a, American Diagnostica) or the isotype-matched control antibodies (clone 679,1MCC7 and U7.27, Beckman Coulter Immunotech) at a final concentration of 10 μg/ml. The cells were washed twice with PBS-1% BSA and incubated 30 min at 4°C with FITC-conjugated polyclonal fragment F(ab')2 anti-mouse IgG (Eurobio). After two washes, samples were analyzed on a Epics XL flow cytometer (Coultronics, Margency, France). Membrane expression levels of uPA and uPAR were determined using calibrated microbeads (QIFIKIT®, Dako) according to the manufacturer’s instructions. Results were expressed as antibody binding sites per cell (ABS/cell). Similar labelling and flow cytometry protocol was used for phenotypic analysis of EPDC, using the following primary monoclonal antibodies: KDR (clone KDR-1, Sigma), CD146 (clone Sendo-1, Biocytex, Marseille, France), CD144, CD34 (clone 581), PECAM-1 (CD31, clone 1F11), CD45 (clone J.33) and CD14 (clone RM052) from Beckman Coulter Immunotech. In some experiments flow cytometry measurement of uPA and uPAR expression level was performed after incubation of adherent cells with 0.9 U/ml of phospholipase C phosphatidylinositol-specific (PI-PLC, Sigma) for 1h at 37°C, as previously described (20).
Enzyme-linked immunosorbent assay
Confluent EPDC and HUVEC were maintained for 24 h in fresh culture medium before collection of cell supernatants. Cells were non-enzymatically detached by EDTA (Sigma), counted and lysed on ice in 100 mM Tris-HCl (pH 8.1), 0.5% Triton X-100 buffer supplemented with complete protease inhibitor mixture (Roche Diagnostic GmbH). The amounts of uPA and uPAR antigens were determined in conditioned media or cell lysates by ELISA according to the manufacturer’s description (894 and 893 Imubind® Elisa kits, American Diagnostica). Results were expressed as ng of uPA or uPAR per 10⁵ cells.

Zymographic analysis
Conditioned media and cell lysates were tested for uPA activity using casein zymography. Samples were normalized according to an equal number of cells (6.10⁵ cells for supernatants and 6.10⁴ for cell lysates) and were run under non-reducing conditions on a 10% SDS-polyacrylamide gel copolymerized with 1 mg/ml ß-casein from bovine milk (Sigma) and 20 µg/ml plasmin-free purified human plasminogen. After washing for 1 h in 2.5% Triton X-100 buffer to elute SDS, gels were incubated in buffer containing 50 mM Tris-HCl pH 8.5, 5 mM CaCl₂, 138 mM NaCl and 0.03% Brij 35 for 20 h at 37°C. Zymograms were developed by staining with Coomassie Brilliant Blue dye and destained to reveal clear bands of casein lysis, indicative of enzymatic activity. The molecular weight of uPA was determined in relation to standard weight markers and purified recombinant mammalian pro-uPA. The specificity of the bands was confirmed by performing gels without plasminogen or incubating gels in lysis buffer supplemented with 1 mM amiloride (Sigma).

For MMP analysis, cells were incubated for 24 h in serum-free EGM-2 medium supplemented with 0.5% bovine serum albumin (BSA, Sigma). Conditioned media were processed for gelatin zymography as previously described (21). Samples were normalized according to an equal number of cells (500). Gelatin type A (1 mg/ml, Sigma) were embedded as substrate in 8.5% SDS–polyacrylamide gel. Conditioned medium of HT1080 cells were used as standard. The nature of the proteases was assessed by using MMP inhibitor 1,10-phenanthroline. After staining, gelatinolytic activities were quantified by densitometry, normalized relatively to HT1080 activities and expressed as arbitrary units. For detection of MMP-9 activity, conditioned media were submitted to gelatin–Sepharose purification (22), and samples were normalized to 2,500 cells.

RNA isolation, cDNA synthesis and real time PCR
Total cellular RNA was isolated from cultured EPDC and HUVEC using RNaseasy Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer’s instructions including Dnase digestion step. Five µg of total RNA were reverse transcribed in a 50 µl reaction containing 40 U RnaseOUT (Invitrogen, Frederick, MD, USA), 150 ng of random hexamer primers (Roche Diagnostics GmbH), 10mM dNTP (Invitrogen), as well as 200 U of Superscript II (Invitrogen). cDNA sample (0.2 µl) was subjected to PCR analysis using primer sets specific for the various genes or control genes at optimized oligonucleotide concentrations of 0.4 mM. Forward and reverse specific primer sequences were: uPA 5’-TTTGGCGGCACTACAGGAG-3’ and 5’- AGTTAAGCCTTGAGCGACC-3’; uPAR 5’-CTGTACACTATTCCGAAGGC-3’ and 5’-AGGTGCTGGTGTGTAGAAC-3’; GAPDH 5’- GGTGGTCTCCCTCCTGA CTCTAAC-3’ and 5’-GTTGCTGTAGCCAAATTCTGTG-3’. Reactions were performed in a total volume of 20 µl using the FastStart DNA MasterPLUS SYBR Green I kit according to the manufacturer’s instructions (Roche Diagnostics GmbH). Amplification cycles were as follows: 10 min at 95°C (hot start PCR), followed by 40 cycles of 10 sec at 95°C, 10 sec at 62°C and 20 sec at 72°C (product amplification). At the end of amplification cycles, melting temperature analysis were performed by slow increase in temperature (0.1°C/sec) up to 95°C. Amplification, data acquisition and analysis were carried out by a LightCycler instrument by means of LightCycler 3.5.2 software (Roche Diagnostics GmbH). This software allows the determination of the threshold cycle (Ct) that represents the number of the amplification cycles where the fluorescence intensity is significantly above the background fluorescence. Ct of the target gene is directly proportional to the log₁₀ of the relative copy number with respect to normalization with parallel GAPDH amplification (to adjust for variations in RNA isolation or cDNA synthesis). The values given refer to the number of specific transcripts detected per 10⁶ relative GAPDH transcripts.

Cell proliferation assay
EPDC or HUVEC (5.10⁵ cells) were seeded on 96-well plates, maintained in EGM-2 medium for 3 days and then incubated for 24 h in EGM-2 or EGM-2 wGF or EGM-2 supplemented with monoclonal antibodies anti-uPA (clone 394OA against uPA B-chain or clone 3471 against uPA ATF fragment, American Diagnostica) or IgG1 isotype control (Biocytex) at 25 µg/ml. The proliferation level was assayed by 5-bromo-2′-deoxyuridine (BrdU) incorporation into cellular DNA using the BrdU Labeling and Detection Kit III from Roche Corporation. In brief, cells were incubated for 12 h with BrdU labelling solution and fixed. Cellular DNA was partially digested by nuclease treatment and incorporated BrdU was detected with peroxidase-conjugated primary antibodies. The absorbance was measured at 405 nm by a micro-plate reader Uvm2 (Safas, Monaco). Results were expressed as a percentage of the absorbance obtained with complete EGM-2 medium considered as 100%.

Wound healing assay
A reepithelialization circular wound was made with a tip on a confluent monolayer of EPDC cultured on 24-well plates. Its surface was measured at 400x magnification under an Olympus inverted microscope coupled with the Biocom Visiolab image analysis software (Les Ulis, France). The medium was removed, and EPDC were incubated for 7 h with EGM-2 or EGM-2 wGF or EGM-2 supplemented with anti-uPA monoclonal antibodies (394OA or 3471 mAb) or IgG1 isotype control, at 25 µg/ml. Wound repair was calculated by subtracting the wound area measured after 7 h of incubation from the area of the original wound. Results were expressed as a percentage of the area of the original wound, considered as 100%.
Endothelial cell tube formation in Matrigel

24-well plates were pre-coated with 1:1 mixture of cold Matrigel™. Basement Membrane (10 mg/ml, BD Biosciences, Bedford, MA, USA) and EGM-2 wGF medium. After 45 min of polymerization at 37°C, EPDC grown for 24 h in EGM-2 wGF were plated at 10⁶ cells/well in EGM-2 wGF supplemented or not with anti-uPA antibodies (394OA or 3471) or IgG1 isotype control at 25 µg/ml. After 6 and 24 h, pictures of 5 representative fields were taken for each condition under an inverted microscope at 400x magnification. Measurement of the number of capillary-like structures and their total length was performed using Lucia® software (Nikon).

Statistical analysis

Data were expressed as mean ± SEM of the indicated number of experiments. Statistical analysis was performed with Prism software (GraphPad Software Inc., San Diego, CA, USA). Significant differences were determined using non parametric Mann Whitney test for comparison between EPDC and HUVEC. Wilcoxon paired test was used for statistical analysis of functional and modulation experiments. A p value of <0.05 was considered significant.

Results

Characterization of EPDC phenotype

Culture of non-adherent MNC from cord blood resulted in the emergence of adherent colonies within 2 to 3 weeks. Cells rapidly expanded into confluent monolayer displaying cobblestone morphology and an uniform endothelial phenotype. They took up Dil-ac-LDL, showed lectin-binding affinity, and expressed specific endothelial markers such as vWF and CD144 (Fig. 1A). No expression of leukocyte markers (CD45, CD14) was detected by flow cytometry analysis of detached cells, demonstrating the absence of contamination of EPDC by monocytic cells (Fig. 1B). After 1 to 6 passages, their uniform endothelial phenotype was maintained, as demonstrated by quantitative flow cytometry analysis of various endothelial antigens such as CD144, CD146, CD31, CD34, and KDR (Table 1). Altogether, EPDC displayed a typical endothelial cell phenotype.

Table 1: Expression level of endothelial and leucocyte antigens on EPDC at passage 2, 4 and 6. Representative results from flow cytometry analysis, expressed as Antibody Binding Sites per cell and percentage of positive cells. Positive expression was considered above 1,000 ABS/cell.

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<td>CD31</td>
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Figure 1: Characterization of EPDC. A) Morphological aspect of endothelial colonies emerging from 2 week culture, of non-adherent MNC from cord blood under endothelial conditions (left picture). Indirect immunostaining and fluorescence microscopy analysis showed that EPDC uniformly expressed vWF (middle picture) and CD144 (right picture, x 1000 magnification). B) Representative histograms, from flow cytometry analysis of detached cells, after immunolabelling with IgG1 control antibody (blue line) versus specific antibodies directed against leukocyte markers (red line), CD45 (left histograms) and CD14 (right histograms).
together, morphology, time of appearance in culture, proliferation potential and phenotype of EPDC were consistent with features of late outgrowth endothelial cells previously described (23–25).

EPDC express higher levels of uPA and uPAR than HUVEC

UPA and uPAR membrane expression levels, determined by quantitative flow cytometry, were significantly higher on EPDC compared to HUVEC (4-fold and 3-fold higher, respectively). These higher expressions on EPDC were maintained from 2 to 6 passages (Fig. 2A, B). In order to investigate whether membrane uPA was only bound to uPAR, uPA and uPAR expression levels were measured after incubation of adherent cells with PI-PLC, a condition previously shown to remove uPAR from cell surface (20). After PI-PLC treatment of both HUVEC and EPDC, the membrane expression of uPA was undetectable while, as expected, the expression level of uPAR was reduced by 80% compared to untreated cells (data not shown), suggesting that membrane uPA was exclusively bound to GPI-anchored molecules.

The uPA and uPAR antigen levels in EPDC and HUVEC lysates were determined by ELISA. Results showed that significantly more uPA and uPAR were present in EPDC than in HUVEC (0.234 +/- 0.066 vs. 0.051 +/- 0.013 ng uPA/10^5 cells, p=0.0286 and 0.518 +/- 0.117 vs. 0.099 +/- 0.036 ng uPAR/10^5 cells, p=0.0317) at passage 2, and unmodified throughout serial passages (Fig. 3A).

Enzymatic activity of cellular uPA was determined by casein zymography in presence of plasminogen. In both EPDC and HUVEC, zymograms revealed the presence of at least two bands of activity. The major band at ~50kD, corresponded to undegraded uPA, migrating in the same position as recombinant pro-uPA used as positive control. Another band, detectable at about 35 kD, corresponds to LMW uPA, a derivative with a slightly degraded A-chain, but intact B-chain containing the catalytic domain. Lysis areas obtained with EPDC, used at passage 2 to 6, were more intense than those obtained with HUVEC, indicating that EPDC displayed and maintained, throughout in vitro expansion, a higher uPA-dependent proteolytic potential compared to HUVEC (Fig. 3C). As controls, no activity was detectable when plasminogen was omitted, or gels were incubated with the uPA inhibitor amiloride (1 mM) (data not shown).
To investigate whether high levels of uPA and uPAR proteins in EPDC were related to a high level of gene transcription, uPA and uPAR mRNA levels were analyzed in both cell types using quantitative real-time PCR (RT-PCR). uPA mRNA level in EPDC at passage 2 was 2-fold higher than in HUVEC, whereas no significant difference was noted in uPAR mRNA levels. (Fig. 3B).

**EPDC secrete higher amount of uPA activity than HUVEC**

The level of secreted uPA protein, quantified by ELISA, was 6- to 10-fold higher in supernatants of EPDC compared to HUVEC (Fig. 3D). Theses differences were significant when EPDC were used at passage 2 (0.783 +/- 0.272 vs. 0.131 +/- 0.036 ng uPA/10^5 cells, p=0.0238), passage 4 (1.596 +/- 0.756 ng uPA/10^5 cells, p=0.0238) and passage 6 (1.068 +/- 0.748 ng uPA/10^5 cells, p=0.0476). Zymographic analysis confirmed that uPA activity was more pronounced in conditioned media from EPDC at passage 2 to 6 compared to HUVEC (Fig. 3E). To test whether gelatinolytic activities could also take part of the proteolytic potential of EPDC, MMP secretion was analyzed using gelatin zymography. A lytic band migrating at 72 kD and corresponding to pro-MMP-2 was detected in EPDC supernatants (Fig. 4A). Densitometry evaluation of the lysis areas revealed that the pro-MMP-2 secretion by EPDC at passage 2 to 6 was significantly enhanced compared to HUVEC (Fig 4B). A low secretion of pro-MMP-9 (lysis band at 92 kD) was also detected in conditioned supernatants from EPDC and HUVEC (data not shown).

**uPA is involved in the angiogenic properties of EPDC in vitro**

The high uPA/uPAR-dependent proteolytic activity of EPDC prompted us to investigate its involvement in the functional properties of EPDC, i.e. proliferation, migration and capillary-like network formation. Treatment of EPDC with antibodies inhibiting either uPA activity (394OA mAb) or uPA binding to uPAR (3471 mAb) significantly reduced cell proliferation (respective proliferation rate of 77.94 +/- 4.959 % p=0.0113 and 80.21 +/- 1.161 % p=0.0034), whereas incubation with control antibody had no effect (97.94 +/- 1.742 %) (Fig. 5A). The effect of uPA Abs was comparable in extent to that induced by growth factor starvation (74.35 +/- 6.524 % p=0.0171).

The impact of uPA inhibition on EPDC migration was then investigated using wound healing assay. Figure 5B shows representative images of EPDC monolayer just after wounding (T0) and after 7 hours (T7) of incubation with isotype control antibody, uPA monoclonal antibodies. The percentage of repair was significantly reduced in the presence of 394OA mAb (16.94 +/- 4.829 %, p=0.0045) or 3471 mAb (16.48 +/- 2.891 %, p=0.0021) compared to control medium (31.44 +/- 4.297 %). A comparable effect was observed by incubating cells in growth factor depleted culture medium (19.08 +/- 3.951 %, p=0.0438). Incubation of EPDC with isotype control antibody did not affect EPDC repair capacity (30.23 +/- 5.012 %) (Fig. 5C).

The formation of capillary tubular structures was evaluated in three-dimensional matrix Matrigel. When EPDC were seeded in the presence of control medium or isotype control antibody, endothelial cells spontaneously organized into capillary-like network. The addition of 394OA or 3471 anti-uPA antibodies resulted in an impairment of network organization. At an early time (6 h), formation of short segments of cords was impaired compared to control conditions (Fig. 6A, left panel). After 24 h, a decrease in capillary-like network area and less closed polygons were observed (Fig. 6A, right panel). Both the number of capillary-like structures and their total length (Fig. 6B, C) were significantly decreased after 24 h incubation with both anti uPA antibodies compared to control. Taken together, these data demonstrated that uPA activity and uPAR binding were involved in the angiogenic properties of EPDC in vitro.

**uPA and uPAR expression by EPDC are differently affected by TNF-α and angiogenic growth factors**

We therefore tested whether EPDC-associated uPA and uPAR expression was modulated by angiogenic growth factors and inflammatory cytokines, both known to be locally secreted during angiogenesis. TNF-α stimulation for 24 h induced a dose-dependent increase in uPA secretion by EPDC. This increase reached statistical significance when TNF-α was used at 50 ng/ml and 100 ng/ml (5.74 +/- 3.48, p=0.078 and 5.96 +/- 4.44 ng of uPA/10^5 cells, p=0.0001, respectively) compared to basal conditions (1.47 +/- 1.742 %) (Fig. 7A). TNF-α (100 ng/ml) also significantly increased uPA secretion by HUVEC although the extent of HUVEC response was lower than that of EPDC (respectively, 3.4 +/- 2.6 and 5.1 +/- 2.9 fold increase compared to basal conditions) (Fig. 7A). At this concen-
Figure 5: Inhibition of uPA by blocking antibodies reduces proliferation and wound repair potential of EPDC. EPDC at passage 2 were incubated in EGM-2 control medium or EGM-2 wGF or EGM-2 supplemented with anti-uPA antibodies (394OA or 3471 mAb) or isotype control antibody (25 µg/ml). Incorporation of BrdU was quantified using a BrdU Labeling and Detection Kit. Results are expressed as percentage of the absorbance at 450 nm obtained with complete EGM-2 considered as 100%, mean ± SEM of 5 separate experiments (A). EPDC repair capacity was measured after wounding of monolayers. Representative fields before and after a 7-hour incubation with antibodies are shown, magnification x 400 (B). The graph summarizes the results of the 5 different experiments, expressed as a percentage of reparation of the original wound area (C). *p<0.05 versus EGM-2 control, **p<0.01 versus EGM-2 control in Wilcoxon paired test.

Figure 6: Inhibition of uPA by neutralizing antibodies impairs capillary-like formation by EPDC in Matrigel. EPDC at passage 2 were seeded on growth factor reduced Matrigel in presence of EGM-2 wGF supplemented by anti-uPA antibodies (394OA or 3471 mAb) or isotype control antibody at 25 µg/ml. A) Capillary-like network formation was observed after 6 (representative fields in the left panel) and 24 hours (representative fields in the right panel, x 400 magnification). Number of capillary-like structure (B) and their total length per fields (C) were quantified by Lucia system on 5 independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus isotype control antibody condition in Wilcoxon paired test.
TNF-α induced significant increase in uPAm RNA levels in both EPDC and HUVEC with peak values observed after 6 and 3 h, respectively (Fig. 7B). TNF-α did not modify uPAR expression by HUVEC or EPDC (Fig. 7C), whereas a significant increase in uPAR transcript level was observed at 6, 12 and 24 h compared to control conditions (Fig. 7D). A 24-h stimulation with 100 ng/ml of TNF-α was not toxic for both cell types as shown by the slight increase in LDH release compared to resting cells (13% +/-4.9 and 14% +/-5.4, respectively), and the proportion of apoptotic and necrotic cells remaining less than 5%.

By contrast, stimulation of EPDC or HUVEC by VEGF or bFGF, at doses ranging from 5 to 100 ng/ml, for 3 to 24 h, did not significantly alter uPA secretion and uPA mRNA levels (data not shown). A dose-dependent increase in uPAR membrane expression level was observed in EPDC stimulated with VEGF for 24 h. This effect reached statistical significance for 50 ng/ml (37244+/12112 ABS/cell, p=0.0442) and 100 ng/ml (34843+/13341, p=0.019) compared to controls (27309+/11339), whereas it could not be observed in HUVEC (Fig. 8A). VEGF (100 ng/ml) also induced a significant up-regulation of uPAR mRNA levels in EPDC (Fig. 8B). In both cell types, uPAR expression and mRNA were not modulated upon bFGF stimulation (Fig. 8C, D).

**Discussion**

The present data show that EPDC, with typical features of late outgrowth endothelial cells, display significantly higher expression of uPA and uPAR than mature endothelial cells. This higher proteolytic potential is maintained during cell amplification and is involved in the angiogenic properties of these cells in vitro.

**In vivo**, EPC contribute to neovascularization by homing to ischemic sites and differentiating into endothelial cells with a potent capacity for vascular repair (8, 26, 27). Transplanted EPC enhance reparative neovascularization in myocardial or limb ischemia whereas differentiated mature endothelial cells do not (28, 29). The molecular mechanisms supporting the advantage of EPC to regenerate vessels or stimulate angiogenesis are incompletely understood. We focused our interest on EPC-derived late outgrowth endothelial cells (9, 24, 25). These cells share a high degree of similarity with mature endothelial cells according to their...
Numerous studies have shown that over-expression of uPA and uPAR induced by growth factors (32), hypoxia (33), uPA gene transfection (34) or related to endothelial tissue specificity (31, 35) resulted in an exacerbated angiogenic phenotype. Thus, among differences between EPDC and mature endothelial cells, the high expression of uPA and uPAR in EPDC may partake of an invasive phenotype supporting their angiogenic behaviour. Consistently, inhibition of EPDC-associated uPA significantly attenuated their proliferation, migration and tube formation in vitro. A similar effect was observed when uPA catalytic activity or uPAR binding to uPAR was blocked, suggesting the involvement of both processes. In various cells with high expression of uPA, proliferation has been shown to be stimulated by endogenous uPA in an autocrine mitogenic mechanism (36), related to both uPA catalytic activity (37) or binding to uPAR and stimulation of mitogenic signaling pathways (38, 39). Interestingly, proliferation of HUVEC was found to be unaffected by inhibition of uPA (data not shown). The high level of endogenous uPA in the EPDC is consistent with the specific participation of uPA in their proliferative activity. Both plasmin mediated pericellular proteolysis and uPAR dependent intracellular signaling pathways have been largely implicated in cell migration and tube formation by endothelial cells (40, 41). Since uPAR also binds to extracellular matrix proteins, the inhibitory effects of anti-uPA antibodies may also be related to interference with adhesion capacity in the leading edge of migrating cells. However, this interference may not be predominant since non-adherent cells counted in cell supernatants were not significantly altered in the

**Figure 8: VEGF increases uPAR membrane expression and uPAR mRNA level in EPDC, whereas bFGF has no effect.** Membrane expression level of uPAR in EPDC and HUVEC, at passage 2, was analyzed by quantitative flow cytometry after 24-hour stimulation by VEGF (A) or bFGF (C) at concentrations ranging from 5 to 100 ng/ml (ABS of uPAR/ cell). Levels of uPAR mRNA, after 3, 6, 12, 24 hours stimulation by VEGF (B) or bFGF (D) at 100 ng/ml, were evaluated by quantitative real time PCR (number of copies/ 10^6 GAPDH copies). Values are mean ± SEM of 5 separate experiments. * p<0.05 versus EGM-2 wGF for EPDC in Wilcoxon paired test.
presence of anti-uPA antibodies. The in vitro model used for evaluation of capillary-like network formation dramatically limited cell proliferation due to short term analysis and growth factor reduced conditions (42). Therefore, the inhibitory effect of uPA antibodies on EPDC reorganization in Matrigel was predominantly related to impairment of proteolysis and cytoskeleton dynamics.

The contribution of the EPC-associated uPA and uPAR in neovascularization has not be directly demonstrated in vivo. However, a recent study pointed out the inhibitory effect of intramyocardial production of plasminogen activator inhibitor on the EPC-mediated neovascularization, indicating that interfering with uPA activity attenuated their angiogenic properties (43). Regarding other proteolytic systems, Urbich, et al. demonstrated that the expression of Cathepsin-L was required for EPC-induced neovascularization in a mouse model of limb ischemia (12). This study was performed on early colonies derived from circulating mononuclear cells that differ from late outgrowth endothelial cells by morphological, phenotypical and functional properties despite a similar capacity of neovascularization in vivo (9, 44). However, these results are of particular interest since Cathepsin-L activates pro-Cathepsin-C which is the specific activator of thrombin-inactivated pro-uPA (45). As further demonstration of the critical role of proteolytic activities in EPC invasiveness, inhibition of MMP, which are downstream from the uPA/plasminogen pathway, significantly attenuated angiogenic properties of EPC in vitro and their incorporation into tumor vasculature (46). These data are consistent with the higher level of pro-MMP-2 secretion also observed in this study. In agreement with a previous report (47), this finding indicates that EPC-derived endothelial cells are equipped with various proteolytic systems that may synergistically contribute to their invasive and angiogenic properties.

The high level of uPA and uPAR expression on EPDC was further increased by mediators known to be locally secreted in ischemic tissues. Interestingly, inflammatory cytokines and angiogenic growth factors had different effects. TNF-α significantly increased uPA secretion and uPA mRNA level in EPDC, a finding consistent with data from mature endothelial cells (48) and the ability of TNF-α to promotes angiogenesis in vivo (49). VEGF, on the other hand, increased uPAR membrane expression and mRNA level in EPDC, as previously reported in human microvascular endothelial cells, whereas bFGF had no effect on uPA and uPAR expression (50). Interestingly, EPDC response to both TNF-α and VEGF stimulation was amplified compared to mature endothelial cells. This observation confirms the higher sensitivity of these cells to growth factors effect as already documented by Bompais, et al. (23). Taken together, these data indicate that EPDC display high levels of uPA and uPAR expression that can be further increased through a cooperation between growth factors and cytokines known to be released into ischemic tissues thereby improving their angiogenic efficiency.

In conclusion, our results showed that EPDC display high proteolytic activity depending on the uPA/uPAR system which is involved in their angiogenic properties in vitro. This particular proteolytic property is a gain for physical incorporation of EPC in newly formed vessels and may reinforce local mature endothelial cell-dependent angiogenesis. Furthermore, preconditioning strategies increasing uPA or uPAR expression during ex vivo expansion might improve the efficacy of EPC transplantation for vascular regeneration.

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