Release of Pro-Inflammatory Mediators and Expression of Pro-Inflammatory Adhesion Molecules by Endothelial Progenitor Cells

Yanmin Zhang1, David A. Ingram2, Michael P. Murphy3, M. Reza Saadetzadeh3, Laura E. Mead2, Daniel N. Prater2 and Jalees Rehman1

1 Section of Cardiology, University of Chicago Pritzker School of Medicine, Chicago, IL and Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, IN
2 Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN
3 Department of Surgery, Indiana University School of Medicine, Indianapolis, IN

Running Title: EPCs and Pro-Inflammatory Mediators

Correspondence to:
Jalees Rehman, MD
University of Chicago
Pritzker School of Medicine
5841 South Maryland Ave, Mail-Code 6080
Chicago, IL 60637
Phone:(773) 834-6687 Fax:(773) 702-2681
Email: jrehman@medicine.bsd.uchicago.edu
Abstract

**Background:** Cell therapy with endothelial progenitor cells (EPCs) is an emerging therapeutic option to promote angiogenesis or endothelial repair. While the release of angiogenic paracrine factors is known to contribute to their therapeutic effect, little is known about their release of pro-inflammatory factors and expression of pro-inflammatory adhesion molecules.

**Methods:** “Early” EPCs and “late” EPCs were isolated from human peripheral blood and their release of chemokines and thrombo-inflammatory mediators as well as their expression of the pro-inflammatory adhesion molecules was assessed at baseline and with stimulation. The effect of Simvastatin on Monocyte Chemoattractant Protein-1 (MCP-1) secretion by “late” EPCs from patients with vascular disease was also evaluated.

**Results:** All groups of EPCs released chemokines and thrombo-inflammatory mediators. “Early” EPCs primarily released thrombo-inflammatory mediators such as Tissue Factor (0.5±0.1 ng/million cells, p<0.05), while adult “late” EPCs primarily released chemokines such as MCP-1 (287±98 ng/million cells, p<0.05). Stimulation with TNF-alpha augmented the expression of pro-inflammatory adhesion molecules and paracrine factors by all EPC subtypes. The release of MCP-1 by “late” EPCs was markedly reduced by Simvastatin treatment of the cells.

**Conclusions:** All EPC subtypes expressed pro-inflammatory paracrine factors and adhesion molecules involved in atherosclerosis. Future clinical studies should therefore not only assess the efficacy of EPCs but also monitor inflammatory activation following EPC transplantation in patients. Pharmacologic modulation of EPCs before and after transplantation may represent a novel approach to improve their safety.

Keywords: endothelial progenitor cells, inflammation, cardiovascular cell therapy,
Introduction

Cardiovascular cell therapy using stem and progenitor cells is a promising novel approach to repair and regenerate cardiovascular cells in patients with cardiovascular disease. Seminal pre-clinical studies demonstrated that adult human blood contained “Endothelial Progenitor Cells” (EPCs) which were able to markedly augment neo-vascularization when transplanted into immune deficient rodents with chronic ischemia (2, 16). While it is known that impaired endothelial function is a prognostic marker for cardiovascular disease (13), multiple studies have now shown that reduced native circulating EPC numbers are not only correlated with reduced endothelial function (15) but also with cardiovascular disease (9, 28), reduced collateral formation (17) and worse long-term cardiovascular outcomes in patients (31). This suggests that replenishment or supplementation of native EPCs could potentially improve endothelial function and cardiovascular outcomes. However, recent larger, controlled clinical studies transplanting EPCs (3) as well as other bone marrow mononuclear cells (21) in patients with myocardial infarction have shown rather limited or even no benefits in overall left ventricular systolic function when compared to the control groups (1).

There is a tremendous diversity in methods and cells that have been employed to study the effects of “EPCs” on cardiovascular function (3, 11, 12, 14-16, 25, 29, 30, 32), and there is a clear need for appropriately defining cells, both for a better mechanistic understanding and for developing clinical therapies (18). There are at least two distinct types of EPCs with apparently distinct properties (11, 12, 14, 15, 25, 29, 32): The first type are referred to as CACs (Cultured Angiogenic Cells) or “early” EPCs because they are obtained from the short-term culture (4-7 days) of circulating adult peripheral blood mononuclear cells and are derived from a myeloid lineage. The second type of EPCs are referred to as “late” or “out-growth” EPCs since they are obtained from the long-term culture (2-4 weeks) of mononuclear cells. These are found to a much greater extent in umbilical cord blood than...
adult human blood, form functional blood vessels, do not express myeloid markers and form highly proliferative endothelial colonies derived from single cells (32). An example for the physiological and clinical significance of distinguishing between EPC subtypes is highlighted by the fact that “late” EPC numbers are higher in patients with coronary disease (12) than control subjects, while “early” EPCs are lower (15). Interestingly, both EPC types appear to use the release of paracrine factors as a key modality by which EPCs enhance angiogenesis (14, 25, 26, 30).

Even though the angiogenic efficacy of “early” and “late” EPCs is well-established, less is known about their potential for harm. A recent in vivo animal study has shown that transplantation of EPCs could potentially promote atherogenesis (10), and concerns have been raised about the balance between safety and efficacy of cardiovascular cell therapy using EPCs or stem/progenitor cells in (5, 23). Since inflammatory activation is a critical component of atherogenesis and endothelial dysfunction (6, 20), we studied the release of pro-inflammatory mediators and expression of surface adhesion molecules in distinct EPC subtypes. We are able to demonstrate that all studied EPC sub-types secrete pro-inflammatory mediators, and can also upregulate pro-inflammatory adhesion molecules upon stimulation in a manner similar to that of mature endothelial cells.
Methods

Isolation and culture of “early” and “late” EPCs

“Early” EPCs were isolated as previously published (25) by culturing human mononuclear cells. These had been obtained after centrifugation of buffy coat preparations from anonymous donors of a blood bank over a Ficoll gradient (25). The mononuclear cells were cultured in the presence of endothelial growth media EGM2-MV, which is endothelial basal medium EBM-2 (Lonza Inc, Walkersville, MD) supplemented with EGM-2-MV-SingleQuots (Lonza Inc, Walkersville, MD) containing vascular endothelial growth factor, basic fibroblast growth factor, insulin-like growth factor-1, epidermal growth factor, and 5% FBS. One million mononuclear cells per cm² were plated on fibronectin-coated tissue culture T-75 flasks (BD Biosciences). As previously published, the mononuclear cells were cultured for four days. Then the flasks were washed twice with PBS to remove the non-adherent cells, while the remaining adherent cells have both endothelial and myeloid characteristics(25, 30).

“Late” EPCs were isolated from either human adult blood or umbilical cord blood by culturing mononuclear cells in endothelial basal medium EBM-2 (Lonza Inc, Walkersville, MD) supplemented with EGM-2 SingleQuots (Lonza Inc, Walkersville, MD) and an additional 10% FBS. Then, 5x10⁶ mononuclear cells/cm² were plated on collagen-coated tissue culture flasks (BD Biosciences). After 1 day of culture, non-adherent cells were discarded, and the media were replaced daily for the next 2-4 weeks. Subsequently, endothelial colonies appeared (on average 1 colony per 10⁷ or 10⁸ plated mononuclear cells). The colonies were re-plated onto new plates and highly proliferative endothelial cells grew out from these colonies which then formed confluent monolayers (32, 33). To confirm the endothelial phenotype, adherent cells were incubated with Dil-labeled acetylated LDL (Molecular Probes-Invitrogen, Carlsbad, CA) for 4 hours, and after fixation they were incubated with the FITC-labeled endothelial surface stain Ulex Europeaus Lectin 1 (Biomedia, Foster City, CA)
for 1 hour, as well as the nuclear DAPI stain (Molecular Probes-Invitrogen, Carlsbad, CA) for 15 minutes(25).

The key differences in the commonly used methods to isolate “early” and “late” EPCs are different culture times of the mononuclear cells (four days for “early” EPCs versus two to four weeks for “late” EPCs, different flask coatings (fibronectin for “early” EPCs versus collagen for “late” EPCs) and different FBS concentrations (5% for “early” EPCs versus total of 15% for “late” EPCs) (25, 30, 32).

The mononuclear cells from which the EPCs were isolated were obtained from anonymous blood bank donors or volunteer subjects and the study was approved by the Human Subjects Institutional Review Board of Indiana University.

To compare the vulnerability of EPCs to that of mature endothelial cells, we obtained mature human aortic endothelial cells (HAECs) from Lonza Inc (Walkersville, MD) and cultured them according to manufacturer instructions.

**Flow Cytometry**

Phenotypic differences between EPC subtypes and the effects of inflammatory stimulation on surface markers were confirmed with flow cytometry. Cells were detached by 2mM EDTA and then incubated with fluorescent antibodies against the myeloid marker CD45 (BD Biosciences, San Jose, CA) and the endothelial marker CD31 (PECAM, BD Biosciences, San Jose, CA) to confirm the origin of these cells. Cells were also labeled with antibodies for the inflammatory endothelial surface marker CD54 and CD106 (ICAM-1 and VCAM-1 respectively, BD Biosciences, San Jose, CA) to assess the response to TNF-alpha stimulation. To control for non-specific antibody binding, all cells were also labeled with a fluorescent negative isotype control antibody. Samples were analyzed on a Becton Dickinson FACSCalibur instrument. The data are presented as fluorescence overlay graphs.
of positive markers and negative isotype controls, as well as quantitative analysis of mean fluorescence.

**Paracrine Activity Assay**

To measure the paracrine activity of all EPCs, cells were placed in basal medium (without serum or potentially contaminating supplemental growth factors) and samples were collected after 24 hours. Since “early” EPCs typically do not proliferate and do not survive long-term, we assessed their paracrine function four days after the mononuclear cells had been isolated and cultured. On the other hand, “late” EPCs had to be isolated by culturing the mononuclear cells for at least 2-4 weeks after which the colonies appeared. The cells were then further cultured and passaged multiple times and the paracrine activity assessment was performed by switching the cells to the same basal medium we used for the “early” EPCs. Levels of chemokines and thrombo-inflammatory mediators in media samples were measured by the Pierce Biotechnology Assay Service, using SearchLight Proteome Arrays (Pierce Biotechnology, Woburn, MA). The SearchLight Proteome Array is a quantitative multiplexed sandwich ELISA containing up to 12 different capture antibodies spotted on the bottom of a 96-well polystyrene microtiter plate. Each antibody captures specific protein present in the standards and samples added to the plate. The bound proteins are then detected with a biotinylated detection antibody, followed by the addition of streptavidin-horseradish peroxidase (HRP) and lastly, SuperSignal™ ELISA Femto Chemiluminescent substrate. The luminescent signal produced from the HRP-catalyzed oxidation of the substrate is measured by imaging the plate using the SearchLight Imaging System which is a cooled charge-coupled device (CCD) camera. The data are then analyzed using ArrayVision™ customized software. The amount of luminescent signal produced is proportional to the amount of each protein present in the original standard or sample. Concentrations are extrapolated off a standard curve. The number of adherent cells was
also quantified, and the data are expressed as secreted ng per 10^6 cells for every paracrine factor over a 24 hour period. To measure whether inflammatory stimulation with the cytokine TNF-alpha (Tumor Necrosis Factor alpha) could augment the release of paracrine activity, cells were exposed to 10 ng/ml TNF-alpha during the 24-hour period in the EBM-2 medium. Change in paracrine activity is shown as a change in the release of the paracrine factor (MCP-1) in ng per 10^6 cells. All results shown reflect n=3 or greater.

The choice of assay factors was based on pro-inflammatory factors that may be involved in the atherogenic process.:

1) **Chemokines** (6): MCP-1 (Monocyte-Chemoattractant-1), IL-8 (Interleukin-8) and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted);

2) **Thrombo-Inflammatory factors** (20): Myeloperoxidase, Tissue Factor and PAI-1 (Plasminogen Activator Inhibitor-1). Statistical analysis was performed by ANOVA using GraphPad Prism software and a value of p<0.05 was considered statistically significant. Following the ANOVA, a Tukey’s post hoc test was performed to compare the paracrine activity of each EPC type with each other. The value for the overall ANOVA and the between group post-hoc analysis are given in the figures and the figure legends. All experiments were performed with n≥3.

**Effect of Simvastatin on EPC paracrine activity**

To assess whether anti-inflammatory treatment of EPCs could mitigate the pro-inflammatory paracrine activity, we chose to treat EPCs with Simvastatin, a commonly used anti-hyperlipidemic agent with known anti-inflammatory effects. Adult “late” EPCs were isolated from mononuclear cells as described above. Cells were switched to endothelial basal medium for 24 hours. To determine whether Simvastatin could modulate the paracrine activity of EPCs, additional cell samples were prepared in which the endothelial basal medium was supplemented with Simvastatin (Sigma Chemicals) or the control vehicle DMSO (dimethylsulfoxide, Sigma Chemicals) during the 24 hour period. The levels of the
pro-inflammatory chemokine MCP-1 were determined by ELISA (R&D Systems, Minneapolis, MN). The number of adherent cells was quantified, and the data are expressed as secreted ng per 10⁶ over a 24 hour period. Statistical analysis of the dose-dependent Simvastatin effect was performed by a repeated measures ANOVA using the GraphPad Prism software. Following the ANOVA, a Tukey’s post hoc test was performed to compare the effect of the varying Simvastatin doses on MCP-1 release.
Results

Distinct subtypes of Endothelial Progenitor Cells (EPC)

Endothelial progenitor cells obtained by the long-term culture of mononuclear cells were able to form proliferative colonies, which stained positive for the endothelial surface membrane stain Ulex europaeus lectin as well as uptake of acetylated LDL (see Fig. 1A). As previously shown, short-term culture of mononuclear cells yields a myeloid cell population (Cultured Angiogenic Cells or “early” EPCs), which also stains positive for Ulex europaeus lectin and has uptake of acetylated LDL (25). The key distinguishing feature of “late” EPCs and “early” EPCs was their surface expression of marker proteins. All EPC types appeared to express the endothelial surface protein CD31 (PECAM), although its expression level was much lower for “early” EPCs (mean intensity of fluorescence 13.2 ± 1.2) than for “late” adult EPCs (mean intensity of fluorescence 186.0 ± 36.0 and “late” cord blood EPCs (mean intensity of fluorescence 185.7 ± 56.9). However, only “early” EPCs expressed the myeloid marker CD45 (mean intensity of fluorescence 33.4 ± 4.2), thus highlighting their hematopoietic origin (see Fig. 1B).

Release of chemokines by EPCs

To investigate whether EPCs released chemokines that are either known to promote inflammatory or atherogenic processes (6), we examined the secretion of the chemokines MCP-1, IL-8 and RANTES by “early” EPCs, adult blood “late” EPCs and cord-blood “late” EPCs (see Figure 2). Interestingly, adult “late” EPCs had the highest release of MCP-1 (“early” EPCs: 6.5±2.5 ng/million cells; adult “late” EPCs: 287.0±98 ng/million cells; cord “late” EPC: 112±18.8 ng/million cells; adult “late” EPCs versus “early” EPCs p<0.01) and IL-8 (“early” EPC: 3.0±0.7 ng/million cells; adult “late” EPCs: 73.4±19 ng/million cells; cord-
blood “late” EPC: 11.3±5.3 ng/million cells; p<0.005). “Early” EPCs had a trend towards the highest release of the chemokine RANTES (“early” EPCs: 8.1±4.0 ng/million cells; adult “late” EPCs: 0.6±0.3 ng/million cells; cord-blood “late” EPCs: 0.1±0.1 ng/million cells; p=0.095).

**Secretion of thrombo-inflammatory factors by EPCs**

Since thrombo-inflammatory factors like Myeloperoxidase, Tissue Factor and PAI-1 can serve as biomarkers and mediators of the thrombo-inflammatory cascade during plaque formation and plaque rupture (20) we measured their release by EPCs (see Figure 3). Myeloperoxidase and Tissue Factor were primarily released by “early” EPCs. The Myeloperoxidase release was 81.5±19.9 ng/million cells in “early” EPCs (p<0.01 versus either adult or cord blood “late” EPCs) and the Tissue Factor release in “early” EPCs was 0.5±0.1 ng/million cells (p<0.05 versus either adult or cord blood “late” EPCs , and their release by “late” EPCs was minimal. However, PAI-1 on the other hand was primarily released by adult-blood “late” EPCs (23933±12011 ng/million cells, p<0.05 versus “early” EPCs) and to a lesser degree by cord-blood “late” EPCs (6017±1510 ng/million cells).

**Changes in EPC paracrine activity induced by TNF-alpha**

Following a therapeutic delivery of EPCs into the cardiovascular system of an atherosclerosis patient, cells could potentially be exposed to pro-inflammatory stimulation since cytokines are commonly up-regulated within the milieu of the vasculature and myocardium (20)To determine if inflammatory stimulation of EPCs could upregulate the release of pro-inflammatory and pro-atherogenic factors, we exposed all three EPC types to the cytokine TNF-alpha and measured the change in paracrine activity of the MCP-1, which plays a key role in the development of atherosclerosis (6, 8). As shown in Figure 4, stimulation with TNF-alpha augmented the paracrine release of MCP-1 in an EPC sub-type
specific manner. "Early" EPCs not only had the lowest baseline release of MCP-1 (Figure 2), but also showed the lowest induction by TNF-alpha (35.5±9.7 ng/million cells), while cord blood “late” EPCs demonstrated the greatest induction of MCP-1 release with inflammatory stimulation (2180±651 ng/million cells, p<0.05 versus “early” EPCs).

**Comparison of inflammatory vulnerability to TNF-alpha between mature endothelial cells and EPCs**

Since a significant component of the atherogenic process involves the upregulation of inflammatory adhesion molecules, we investigated whether EPC subtypes would differ in their inflammatory response, when compared to mature aortic endothelial cells. “Early” EPCs did not express the adhesion molecule VCAM-1 at baseline (mean fluorescence: 4±0.1) or with stimulation (mean fluorescence: 5±0.2). Adult and cord blood “late” EPCs demonstrated a significant degree of VCAM-1 upregulation with TNF-alpha stimulation: A) adult baseline: 9±1.2 and adult stimulation: 402±1.2, B) cord baseline: 6±0.8 and cord stimulation: 307±77 (representative flow cytometry histograms in Fig. 5A). The “late” EPC response was comparable to that of mature endothelial cells (mean fluorescence at baseline 8±0.8 and with stimulation 216±38). The surface adhesion molecule ICAM-1 was expressed on all studied cells at baseline, but upregulated to a much greater extent on “late” EPCs or mature endothelial cells than on “early” EPCs (Fig. 5).

**Modulation of pro-inflammatory paracrine activity with Simvastatin**

Adult “late” EPCs were cultured and their release of the pro-inflammatory chemokine MCP-1 was measured with or without the presence of Simvastatin. Since statins do not just control lipids, but also have anti-inflammatory effects on the mature endothelium (22), we evaluated whether addition of Simvastatin to the cultured EPCs was able to reduce the release of MCP-1 by EPCs. As shown in Fig. 6, when EPCs were treated with Simvastatin, they
showed a marked reduction of MCP-1 release of up to 70% with addition of 1µM Simvastatin (p<0.01 versus control).
Discussion

The paracrine activity of EPCs

The release of paracrine factors by stem and progenitor cells in the setting of cardiovascular cell therapy appears to be a major underlying component of the therapeutic effect (7, 14, 25, 26, 30). Furthermore, EPCs are thought to release multiple synergistic, therapeutic angiogenesis factors (7, 25) which may explain the potent neovascularization observed in animal models after EPC transplantation. However, it was recently shown in a pre-clinical study that EPC transplantation can also promote the growth of atherosclerotic plaques (5, 10) and concerns have been raised about potential risks of cardiovascular stem and progenitor cell therapy (5, 10). Little is known about the release of pro-inflammatory factors by EPCs and whether the various EPC subtypes that are frequently used in animal models and patients differ in their ability to release pro-inflammatory cytokines.

We therefore studied the chemokines MCP-1, IL-8 and RANTES, which have all been shown to be involved in the growth and formation of the atherosclerotic plaque by recruiting circulating inflammatory cells (6, 8). Since thrombus formation and plaque rupture is another major aspect of cardiovascular disease, we also studied thrombo-inflammatory factors like Myeloperoxidase, Tissue Factor and PAI-1, which are biomarkers and mediators of the thrombo-inflammatory cascade during plaque formation and plaque rupture (20). Our study focused on identifying potential inflammatory factors that could mediate such pro-atherogenic effects of EPCs. The key finding of our study are that the studied types of EPCs have a distinct pattern of chemokine and thrombo-inflammatory factor release. Additionally, we were able to show that the paracrine release is significantly augmented when EPCs are exposed to the pro-inflammatory cytokine TNF-alpha and that TNF-alpha can induce similar degree of surface adhesion molecule upregulation in EPCs as in mature endothelium.

Patients requiring EPC therapy due to endothelial dysfunction or chronic coronary artery
disease are likely to contain pro-inflammatory cytokines such as TNF-alpha in their vasculature (20). Based on our results, it appears that transplanted EPCs would be vulnerable to an inflammatory environment. While it is important for transplanted cells to maintain their physiologic ability to respond to stimuli, our findings highlight that it may be necessary to monitor the inflammatory activity following EPC transplantation and assess if transplanted cells are participating in pathological inflammatory processes.

**Finding the ideal stem or progenitor cell for cardiovascular cell therapy**

A major finding in our study was that all three studied cell types released pro-inflammatory factors, and that the paracrine profile was distinct for “early” EPCs versus “late” EPCs. A cell-type specific function and phenotype profile may need to be established for each cell type and matched with a specific therapeutic goal such as post-infarct repair versus collateral formation. Recent arteriogenesis studies are raising the importance of individualizing therapeutic approaches based on the patient’s need and micro-environment(27). Monocyte/macrophages play key roles in the post-infarct repair by limiting infarct extension(19) and in collateral formation(8). It may be the pro-inflammatory monocytic-myeloid character of “early” EPCs that is responsible for their therapeutic effects in the setting of post-infarct repair or collateral formation(4, 23) and may, for example, make them ideal cell therapies in the post-infarct setting. However, due to their pro-atherogenic paracrine activity, they may not be the optimal choice to restore endothelial function and reduce atherosclerosis progression.

**“Janus” Phenomenon in Cardiovascular Cell Therapy: Safety vs Efficacy**

Pre-clinical studies using angiogenesis gene therapy have been highly successful in animal models but have had more modest benefits in the clinical setting. One reason for the
limited success in translating angiogenesis therapies to the clinical setting could be the “Janus” phenomenon(8). It is a term used to describe the intricacy in approaching therapeutic angiogenesis, as many of the factors involved in desired collateral growth are also involved in the growth of the atherosclerotic plaque(8). One such example is the chemokine MCP-1, which is an extremely potent arteriogenic factor, but is also thought to promote vascular inflammation and atherosclerosis(8). EPCs release a synergistic cocktail of angiogenic factors which (25) may account for their considerable therapeutic effects, but our work now demonstrates that they also release a combination of detrimental factors that could result in a negative synergy, affecting plaque growth and rupture or promote a pro-inflammatory environment in the vasculature, and possibly also damaging the myocardium.

The role of pharmacotherapy in modulating EPC paracrine activity

We examined the release of MCP-1, which appears to be a key mediator in atherosclerosis progression as well as plaque rupture (6), from “late” EPCs in the presence of Simvastatin. We chose to study “late” EPCs, since a recent study confirmed that the “late” EPCs have long-term growth potential and can form actual vascular tubes, thus making them candidates for future therapies targeting vascular tissue engineering (29). We found that the release of the pro-atherogenic chemokine MCP-1 could be markedly reduced by co-culturing the cells with Simvastatin, a known anti-inflammatory agent in endothelial cells (22). This finding points to an important novel therapeutic option in cardiovascular cell therapy: The use of anti-inflammatory agents to treat cells prior to cell transplantation and thus improve their safety. Our study only investigated Simvastatin, but future studies could evaluate other statins as well as other anti-inflammatory agents during the cell culture period, with the specific goal to reduce the pro-inflammatory potential of cells. Clinical studies using cell therapies could also consider modifying the therapeutic regimen in patients in the peri-transplantation time period to improve the safety of cardiovascular cell transplantation.
**Limitations**

All our assessments of cell activity were performed on cultured EPCs, because that is how they are currently defined and isolated (11, 29, 32). The culture process and differences in culture protocols for “early” and “late” EPCs also make it difficult to make direct inferences regarding possible native inflammatory function of the circulating cell populations that give rise to the cultured “early” and “late” EPCs. Furthermore, adult “late” EPCs may also contain some mature endothelial cells and the culture process can result in maturation of EPCs to more mature endothelial cells.

Therefore, our focus was not to understand the physiology of circulating EPCs, but instead what potential risk cultured EPCs may harbor when transplanted into patients. As the analysis was performed on a small group of patients, we could not extensively analyze subgroups and the effects of risk factors on the paracrine activity. However, by identifying some of the pro-inflammatory factors released by cultured EPCs, we are providing the tools for future large-scale trials to monitor serum levels in patients who are recipients of EPC transplantation. Since a recent study demonstrated in an animal model that factors released by transplanted EPCs can be measured in the serum (7), future clinical trials could monitor the appropriate serum factors in patients. The EPC sub-type specific chemokines, adhesion molecules and thrombo-inflammatory markers identified in this study could serve as a starting point to monitor inflammatory activation in patients following EPC transplantation. This approach will ultimately allow for greater safety of patients receiving cell therapy. While we chose specific representatives of the class of chemokines and thrombo-inflammatory factors for our study and based our choice on some factors that have a well-established role in atherosclerosis, it is possible and likely, that EPCs also release multiple additional factors which play a major role in atherosclerosis.
In conclusion, cardiovascular cell therapy with EPCs needs to be accompanied with the necessary caution and monitoring of possible inflammatory activation in the host vasculature to prevent the development of pathological vascular inflammation. Furthermore, there is an emerging recognition that angiogenic therapy needs to be individualized for each patient (24), and our findings demonstrate that paracrine profiling of cells may also allow for a more optimal choice of EPC subtype. If the release of chemokines by EPCs is of significant concern in a selected patient group, pharmacologic treatment of cells (and possibly patients) during the peri-transplantation period may modulate the release of pro-inflammatory chemokines. Careful matching of specific therapeutic goals with well-defined and appropriate cell therapies will allow for achieving an optimal balance between therapeutic effects and the risk of side effects such as atherogenesis and plaque rupture.

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**Disclosures:** Dr. David Ingram owns stock in EndGenitor Technologies, Inc (Indianapolis, IN). He has also acted as a consultant to and member or officer of the Board of EndGenitor Technologies within the last 3 years.
References


**Figure Legends**

**Fig 1:** A) Phase contrast and fluorescent images of a cord blood-derived “late” EPC (endothelial progenitor cell) colony. Endothelial cells derived from the long-term culture of mononuclear cells (at least 2 to 4 weeks) can form highly proliferative colonies derived from single circulating endothelial cells. The left side of this panel shows a phase contrast image of an EPC colony derived from umbilical cord blood mononuclear cells, while the right side shows a confocal microscopy image of an endothelial colony stained with the endothelial surface stain *Ulex europaeus* Lectin (green=FITC label), the nucleus stain DAPI (blue) as well as uptake of acetylated LDL (red=Dil label). B) **Flow cytometry phenotyping of EPCs using the endothelial surface marker CD31 (PECAM) and the myeloid surface marker CD45.** “Late” EPCs (or colony-forming EPCs) derived from the long-term culture (at least 3-4 weeks) of circulating mononuclear cells from adult blood and umbilical cord blood, are positive for the endothelial marker CD31 (PECAM) but not for the myeloid marker CD45; while “early” EPCs (or CACs) derived from the short-term culture (4 days) of adult mononuclear cells, also express the myeloid marker CD45.

**Fig 2:** Release of inflammatory chemokines by EPCs. Release of the chemokines MCP-1 (Panel A, Monocyte-Chemoattractant-1), IL-8 (Panel B, Interleukin-8) and RANTES (Panel C) by “early” EPCs (n=4), adult blood “late” EPCs (n=3) and cord-blood “late” EPCs (n=4) was measured over a 24 hour period. The data for each chemokine are presented as mean ± standard error of mean of secreted ng per million adherent cells. In Panel A, the overall p-value for comparative MCP-1 release by ANOVA was p<0.01, but the only significant difference in the post-hoc analysis was p<0.01 (shown as **) between “early” EPCs and adult “late” EPCs. In Panel B, the overall ANOVA of the IL-8 release was highly significant (p<0.005), and this was driven by the difference in IL-8 release between “early” EPCs and adult “late” EPCs (p<0.01, shown as **) and by the difference between cord-blood “late”
EPCs group and adult “late” EPCs (p<0.01, shown as **). In Panel C, the overall ANOVA of the RANTES release was not significant (p=0.095), so no post-hoc analysis could be performed, although there was a trend towards higher RANTES release by “early” EPCs when compared to either one of the “late” EPC groups.

**Fig 3: Release of thrombo-inflammatory factors by EPCs.** The release of the thrombo-inflammatory factors Myeloperoxidase (MPO, Panel A), Tissue Factor (Panel B) and PAI-1 (Plasminogen Activator Inhibitor-1, Panel C) by “early” (n=4), adult blood “late” EPCs (n=3) and cord-blood “late” EPCs (n=4) is shown over a 24 hour period. The data for each factor are presented as mean ± standard error of mean of secreted ng per million adherent cells. In Panel A, the overall p-value for comparative MPO release by ANOVA was p<0.005, and post-hoc analysis revealed that “early” EPCs had markedly higher MPO release (p<0.01 shown as **) compared to either adult or cord-blood “late” EPCs, both of which showed only minimal MPO release. The release of Tissue Factor (Panel B) was also markedly higher in “early” EPCs (p<0.05 versus either of the two “late” EPC groups), while there was no significant difference in Tissue Factor release between the two “late” EPC groups. In Panel C, the release of PAI-1 is shown, and statistical analysis demonstrated that adult “late” EPCs had significantly higher PAI-1 release (p<0.05) than “early” EPCs, and a trend towards higher PAI-1 release when compared to cord-blood “late” EPCs.

**Fig 4: Changes in EPC paracrine activity induced by TNF-alpha.** To assess whether inflammatory stimulation of EPCs could modulate their paracrine activity, EPCs were exposed to 10 ng/ml of the pro-inflammatory cytokine TNF-alpha during a 24 hour period. The concentration of the pro-inflammatory chemokine MCP-1 was determined and the change in paracrine activity was expressed as the difference in released MCP-1 ng per million cells between baseline and after TNF-alpha stimulation. The data is presented as mean ± standard error of mean of secreted ng per million adherent cells. Statistical analysis
is performed as a between group ANOVA on the degree of change in MCP-1 release and post hoc analysis revealed that “early” EPCs (n=4) had minimal induction of MCP-1 release by TNF-alpha (p<0.05) when compared to cord blood “late” EPCs (n=4), while adult “late” EPCs (n=3) had an intermediate increase in MCP-1 release.

Fig 5: Cell surface expression of pro-inflammatory cell adhesion molecules VCAM-1 (CD106) and ICAM-1 (CD54) on Endothelial Progenitor Cells

Representative flow cytometry histograms of VCAM-1 expression on “early” EPCs, adult blood derived “late” EPCs and cord blood derived “late” EPCs at baseline and with TNF-alpha stimulation (20 hours, 10 ng/ml) are shown in Panel A. All three EPC subtypes express minimal VCAM-1 at baseline, however only “late” EPCs show a marked upregulation of VCAM-1, a surface marker characteristic of endothelial inflammation. The quantitative assessment of pro-inflammatory surface adhesion molecule expression demonstrates that “early” EPCs upregulate ICAM-1 (Panel C) but not VCAM-1 upon stimulation (Panel B). Due to the massive response to the stimulation, the mean fluorescence of ICAM-1 and VCAM-1 is shown on the y-axis on a log scale. “Late” EPCs (both adult and cord blood derived) have a similar response to inflammatory stimulation as mature human aortic endothelial cells (HAECs), thus highlighting their physiologic similarity.

Fig 6: Secretion of the Pro-Inflammatory Chemokine by Endothelial Progenitor Cells treated with Simvastatin:

Adult-blood derived “late” EPCs were cultured with increasing doses of Simvastatin to assess its effect on the release of MCP-1. Released MCP-1 was assessed by ELISA and normalized by cell number. The data is shown as mean ± standard error of mean of secreted MCP-1 in ng per million cells. Cells showed significant decreases in their release of MCP-1.
per repeated-measures ANOVA, n=4 with Simvastatin treatment (n=4, post hoc analysis showed that 1µM Simvastatin markedly reduced MCP-1 release (p<0.01 versus control shown as ** ).
Fig 1

A

B

PECAM Expression

C

CD45 Expression

B

C

Fig 1
Fig 3

A

Myeloperoxidase

<table>
<thead>
<tr>
<th>Group</th>
<th>ng per 10⁶ cells</th>
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<td>Cord Blood Late EPCs</td>
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p<0.005

B

Tissue Factor

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<tr>
<td>Adult Blood Late EPCs</td>
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<tr>
<td>Cord Blood Late EPCs</td>
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p<0.05

C

PAI-1

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<td>Cord Blood Late EPCs</td>
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p<0.05
Figure 4

Increase in MCP-1 by TNF-alpha

- Early EPCs (CACs)
- Adult Blood Late EPCs (Adult CF-EPCs)
- Cord Blood Late EPCs (Cord CF-EPCs)

Change in secreted MCP-1 per $10^6$ cells

*p<0.05*
Fig 5
Simvastatin Reduces MCP-1 Release by Adult Late EPCs

Fig 6