Full Length Research Paper

Potential proliferative effect of lipopolysaccharide preconditioning on human umbilical cord blood-derived endothelial progenitor cells

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Endothelial progenitors cells (EPCs) are important for the development of cell therapies for various diseases. However, the major obstacles in developing such therapies are low quantities of EPCs that can be generated from the patient and the lack of adequate non-invasive imaging approach for in vivo monitoring of transplanted cells. Bacterial lipopolysaccharide (LPS) is a key mediator in the vascular leak syndromes associated with gram-negative bacterial infections. Toll-like receptor 4 (TLR4) was expressed both on the surface and intracellular compartment of EPCs. The objective of this study was to determine the ability of human umbilical cord blood (hUCB) EPCs to be isolated and identified in vitro and examine the effect of LPS on EPCs- TLR4 signalling pathway. 5 hUCB samples were collected for EPCs isolation and cultured on fibronectin coated wells in endothelial media supplement. Their phenotypes were confirmed by uptake of acetylated LDL and binding of fluorescein isothiocyanate (FITC) - labeled Ulex europaeus agglutinin 1 (UEA-1) lectin. LPS was added to EPCs culture by different concentrations (0.1, 0.5 and 1 µg/ml) to assess its effect on EPCs proliferation. The following genes; human vascular endothelial growth factor receptor-2 (hVEGFR-2), TLR4, vascular endothelial-cadherin (VE-Cadherin), endothelial nitric oxide synthase (eNOS) and heme oxygenase (HO-1) from the culture EPCs (control group) and EPCs preconditioned with 0.1 µg/ml LPS (preconditioned treated group) were also assessed by Real-time qPCR. EPCs cultured from hUCB were isolated, identified and highly significant proliferated in response to 0.1 µg/ml LPS compared to other concentrations. The expression of hVEGFR-2, TLR4, VE-Cadherin, eNOS and HO-1 by EPCs preconditioned with LPS compared to EPCs was significantly increased in response to the toll-like receptor agonist LPS. hUCB is highly valuable rich source for EPCs. Data of Quantitative genes expression indicate that LPS recognition by EPCs-TLR4 activates the corresponding signal pathway.

Key words: Human umbilical cord blood (hUCB), Endothelial progenitors cells (EPCs), lipopolysaccharide (LPS), Toll-like receptor 4 (TLR4).

INTRODUCTION

Endothelial progenitor cells (EPCs) have been demonstrated to have stem-cell like as well as mature endothelial functions (Russell and Brown, 2014). In 1997, Asahara and colleagues identified a monocytic population
of adult human CD34+ cells that demonstrated clonogenicity as well as contributed to neovascularization within ischemic areas (Asahara et al., 1997). EPCs are thought to be recruited through the circulation by an incompletely defined cytokine-mediated pathway to sites of vascular injury or hypoxia. In addition to self-renewal, EPCs differentiate into mature endothelial cells and release proangiogenic cytokines and growth factors in order to form new blood vessels and/or incorporate into existing vasculature (Folkman, 1971; Bertolini et al., 2006; Resch et al., 2012). As circulating EPCs mature, the expression of endothelial-specific markers (VE cadherin, E-selectin) increases, and CD133 expression may decline. Accordingly, circulating EPCs can be characterized by the expression of CD133 (for developmentally immature EPCs), CD34, KDR, and/or VE cadherin (Shintani et al., 2006). Human umbilical vein blood derived EPCs expressed gene transcripts coding for Toll-like receptor (TLR). Toll-like receptors (TLRs) are a class of molecules which play an important role in the innate immune system for the recognition of pathogen-associated molecular patterns by immune cells, initiating a primary response toward invading pathogen and recruitment of the adaptive immune response (Hoebe et al., 2004).

Lipopolysaccharide (LPS) is the antigenic component of the gram-negative bacterial cell wall and is known as the ligand of Toll-like receptor 4 (TLR4). The activation of TLR4 by LPS modulated the expression of TLRs, induced the phosphorylation of NF-kappaB, and up-regulated the gene expression of cytokines IL-8, IFN-alpha, IFN-beta, and TNF-alpha, suggesting EPCs expressed functional TLR4. Unexpectedly LPS treatment failed to induce apoptosis in EPCs, but instead promoted cell proliferation of EPCs. Furthermore, the treatment of EPCs with LPS up-regulated stem cell markers CD133 and CD34 in both mRNA and protein levels. These results suggested that TLR4 functions to maintain the stem cell phenotype of EPCs and enlarge its population (He et al., 2010). We investigated in this work that, the effect of LPS as TLR4 agonists on the proliferation and maintenance of EPCs function in culture for future prospects using TLR4 agonists in promoting proliferation of EPCs in clinical trial uses.

MATERIALS AND METHODS

Human cord blood sample

Twenty ml human umbilical cord blood (HUCB) was collected. The HUCB mononuclear cell fraction (HUCB-MNCs) was isolated from theuffy coats through density-gradient centrifugation with Ficoll-Paque (Gibco-Invitrogen, Grand Island, NY). Anticoagulated blood sample was carefully layered on 20 ml Ficoll, and then they were centrifuged for 35 min at 400 x g rpm. The upper layer was aspirated leaving the HUCB-MNC layer undisturbed at the interphase. The interphase layer HUCB-MNC layer was carefully aspirated and washed twice in PBS containing 2 mM EDTA and centrifuged for 10 min at 200xg rpm at 20°C. The cell pellet (3 x 10^6) was resuspended in a final volume of 300 μl of buffer (Matsuo et al., 2008).

EPCs culture, propagation, labeling for identification and counting based assay

EPCs were identified in culture by formation of a Colony Forming Unit (CFU). CFU was defined as a central core of rounded cells surrounded by elongated spindled-shaped cells, and CFUs were formed after culturing HUCB-MNCs for 7 days. EPCs-CFU was used as a method for EPCs characterization. For the EPCs counting assay, 5 x 10^6 MNCs were layered onto fibronectin coated 96-well plates and cultured in M199 medium supplemented with 20% fetal calf serum (FCS), 0.1% human vascular endothelial growth factor-1 (VEGF-1) and 0.1% insulin-like growth factor (IGF-1) at 37°C for 48 h, after which the supernatant was removed. After seven days, cells were stained and labelled with 1, 1'-dioctadecyl-3, 3', 3'-tetramethylindocarbocyanine-labeled acetylated LDL (DILDL,) and FITC-labelledUlex europeus agglutinin I (UAE-1, Sigma Chemical Company); this double staining is specific for EPCs identification. Cells were counter stained with 4’, 6-diamidino-phenylindole (DAPI; 0.2 μg/ml in 10 mmol/l Tris-HCl, pH 7.0, 10 mmol/l EDTA, and 100 mmol/l NaCl) for 10 min and cells visualized with a distinct blue cytoplasm under inverted fluorescent microscope; DAPI staining is used to ensure cells viability. Only double stained cells (DILDL-FITC labelled UAE-1) with a distinctly blue cytoplasm (DAPI positive cells) were counted in a five random fields under fluorescent inverted microscope (Lieca, Germany).

Cell culture and cell proliferation assay

LPS was dissolved in complete DMEM, the pH value adjusted to 7.2 and sterilized through a 0.2 μm filter to the desired working solutions. EPCs were cultured in M199 medium supplemented with 5% fetal bovine serum (FBS), 100 mg/mL streptomycin and 100 units/mL penicillin at 37°C humidified atmosphere 5% CO2. EPCs were seeded in 96-well plates (10^3 to 10^5 cells/well) for 24 h incubation, cell viability was evaluated using MTT assay as described previously (Cui et al., 2007). Brief, cells were treated with LPS at various concentration 0, 0.1, 0.5 and 1 μg/ml for 48 h and untreated cells served as a control. Prior to determination, 10 μL MTT (2.5 g/L) was added to each well. After 4 h incubation, the culture media were discarded followed by addition of 100 μL of detergent reagent to each well and vibration for 10 min. The absorbance (A) in the experimental wells was measured at 570 nm with a microplate reader (ELISA reader). The absorbance in the experimental wells to that of the control wells (without test compound).

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Abbreviations: EPCs, Endothelial progenitors cells; LPS, lipopolysaccharide; hUCB, human umbilical cord blood; FITC, fluorescein isothiocyanate; TLR4, toll-like receptor 4; hVEGFR-2, human vascular endothelial growth factor receptor-2; TLR4, VE-Cadherin, vascular endothelial-cadherin; eNOS, endothelial nitric oxide synthase; HO-1, heme oxygenase; LDL, low-density lipoprotein.

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Table 1. Primers sequence for the studied genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEG-R2</td>
<td>CATGTCGTGTTCTGAGTCCGTCT</td>
<td>CATGGGTCTGTCTTCTCCTTTG</td>
<td>NT_022853.15</td>
</tr>
<tr>
<td>eNOS</td>
<td>ATTATATCTACCAAGACTCCAG</td>
<td>TTTCAAGTTGCCATAGTTAC</td>
<td>NT_007914.15</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>TCCCTCTGACCTCCACGACCTCA</td>
<td>TAAATGACCAACTGCTCGTGAAT</td>
<td>NM_001795.3</td>
</tr>
<tr>
<td>TLR4</td>
<td>TGCTACGTTGTCCTTTAAAT</td>
<td>GAATGGAAGGACCCCTTC</td>
<td>NM_138557.2</td>
</tr>
<tr>
<td>HO-1</td>
<td>CAGGCAAGAGAAATGCTAGGTC</td>
<td>GCTTCCACATAGCGCTGCA</td>
<td>X06985.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CTCCTACTGGCGCTGGGAACAT</td>
<td>GTCCACACTGACACGTTG</td>
<td>NT_009759.16</td>
</tr>
</tbody>
</table>

Figure 1. **A**, EPCs at 0 day of culture; **B**, EPCs were more confluent in culture on 4th day (200X magnification).

Analysis of EPCs based on cell surface marker expression

Flow Cytometry Analysis (FACS analysis) for KDR and CD34+ of cultured EPCs was done as an identification surface marker of EPCs. Mononuclear fractions (4 × 10⁶ cells) were incubated with FcR blocking reagent (MiltenyiBiotec) for 10 min, and then incubated with CD34-FITC (MiltenyiBiotec). Dead and dying cells were excluded with 7-AAD (1 μg/10⁶ cells, Molecular Probes). Isotype control antibodies were used to set baseline fluorescence levels.

QRT-PCR genes expression to assess EPCs function

Total RNA was isolated from collected cultured human EPCs using Qiagen cells/tissue extraction kit (Qiagen, USA) according to instructions of manufacture. The purity (A260/A280 ratio) and the concentration of RNA were obtained using spectrophotometry (dual wave length Beckman, Spectrophotometer, USA). The extracted and purified RNA samples were treated with RNase free DNase at 37°C for 20 min and stored at -80°C for further use. Sequences of primers were designed in Table 1. The total RNA (0.5 to 2 μg) was used for cDNA conversion using high capacity cDNA reverse transcription kit Fermentas, USA. The cDNA 25 μl master mix was prepared; first strand buffer (10x) 5 μl, 10 mM dNTP’s, RNase inhibitor (40 U/μl), MMLV-RT enzyme (50 U/μl), and DEPC-treated water. The last mixture was incubated in the programmed thermal cycler 1 h at 37°C followed by inactivation of enzymes at 95°C for 10 min, and finally cooled at 4°C. Then cDNA was stored at -20°C. Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets (Table 1) were optimized at the annealing temperature. All cDNA including previously prepared samples (for VEGFR-2, eNOS, VE-cadherin, TLR4 and HO-1 genes expression), internal control (for GAPDH gene expression as housekeeping gene), and non-template control (water to confirm the absence of DNA contamination in the reaction mixture), were in duplicate. Each 25 μL of reaction mixture contained 12.5 μL of SYBR Green (Fermentas), 1 μL of each primers (10 μmol/L), and cDNA (1 μg/mL) for sample determination. The reaction was initiated by activation of Taq polymerase at 95°C for 5 min, followed by 40 two-step amplification cycles: 10 s denaturizing at 95°C, 50 s annealing at 52°C. After the RT-PCR run the data were expressed in Cycle threshold (Ct) of assessed genes (VEGFR-2 and eNOS) and the house keeping gene (GAPDH). Therefore, relative quantitation (RQ) of target genes expression was assessed and related to housekeeping gene by previously published method RQ = 2^-ΔΔCt (Pala et al., 2005; Jiang et al., 2008).

Statistical methods

The data of the study was presented as mean ± SD. SPSS 15.0 software (SPSS Inc.) and was used to analyze the data. The chi-square test was used for comparisons of categorical variables. ANOVA was used to determine significant differences between different groups. Significant differences were considered when P value < 0.05.

RESULTS

EPCs isolation, culture and propagation of human umbilical cord blood EPCs were assessed. Characterization and labelling of EPCs by DiLDL-UEA 1-lectin double fluorescent staining were done. EPCs were rounded in their shape and adherent on fibronectin plate (Figure 1). EPC-CFU as a central core of rounded cells surrounded by elongated spindled-shaped cells cultured on fibronectin plate (Figure 2). Adherent double-stained cells...
Figure 2. EPC-CFU. A. at 24 h culture less confluent was seen (200X magnification). B. at 7 days of culture more confluent was seen (200 X magnification).

Figure 3. Specific DiLDL-UEA 1 lectin double staining of EPCs cultured 7 days for characterization. A: 200X magnification and B: 400X magnification.

Figure 4. Characterization of viability of 7 days cultured EPCs by positive-DAPI blue cytoplasm staining (200X magnification).

(DiLDL-UEA 1-lectin) were identified as EPCs (Figure 3). Staining of nuclei with DAPI for nearly all adherent cells (>95%) were stained positive for both DiLDL-UEA 1-lectin (Figure 4). As regard cell proliferation assay results; significant difference was detected between zero (0) concentration and other studied concentration. The highest significant difference was found at 0.1 µg/ml compared to 0.5 and 1 µg/ml (Table 2 and Figure 5). Results of quantitative genes expression by real time PCR show that EPCs treated with LPS (0.1 µg/ml) studied groups have significant difference (p value =0.001) from untreated EPCs (Tables 3).
Table 2. Cell proliferation assay for EPCs treated with different concentrations of LPS.

<table>
<thead>
<tr>
<th>Different group</th>
<th>EPCs</th>
<th>EPCs + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LPS (0.1 µg/ml)</td>
</tr>
<tr>
<td>Cell proliferation (mean ± SD)</td>
<td>0.156±.065</td>
<td>0.638±.161</td>
</tr>
<tr>
<td>P1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>P2</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>P3</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

P1: Comparison between zero (0) concentration and other 3 different concentrations. P2: Comparison between 0.1 µg/ml concentration and other three different concentrations. P3: Comparison between 0.5 µg/ml concentration and other 3 different concentrations.

Figure 5. Cell proliferation assay in different studied groups (* means significant difference between different groups).

Table 3. Showed quantitative expressions of all target genes in the studied groups.

<table>
<thead>
<tr>
<th>Gene expression/10^3-10^5 cells/ml</th>
<th>Group 1 (Untreated EPCs)</th>
<th>Group 2 (EPCs treated LPS)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>e NOS</td>
<td>5.13 ± 1.13</td>
<td>6.50 ± 1.54</td>
<td>0.001</td>
</tr>
<tr>
<td>TLR4</td>
<td>4.38±0.81</td>
<td>6.91±1.08</td>
<td>0.001</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1.31±0.46</td>
<td>4.10±1.15</td>
<td>0.000</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>0.39±.21</td>
<td>2.23±1.08</td>
<td>0.000</td>
</tr>
<tr>
<td>HO-1</td>
<td>2.33±.34</td>
<td>4.83±5.2</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The data are mean ±S.D.

DISCUSSION

EPCs described in 1997 by Asahara et al. constitute a heterogeneous population of circulating cells in peripheral blood. Endothelial progenitor cells play an important role in vascular repair and new vessel formation, because of their capacity to proliferate, migrate, differentiate in vivo and in vitro into endothelial cells, and incorporate into the preexisting endothelium. Thus, phenotypically, they have morphofunctional characteristics of both hematopoietic and mature endothelial cells (Silva et al., 2012). The common barrier for the characterization and subsequent utilization of putative EPCs is the poor number of cells obtained after purification from peripheral or cord blood.
EPCs are characterized by the assessment of surface markers such as CD34 and vascular endothelial growth factor receptor-2, VEGFR-2 (KDR). Importantly, CD34‘KDR’ combination is the only putative EPC phenotype that has been demonstrated repeatedly and convincingly to be an independent predictor of cardiovascular outcomes (Devaraj and Jialal, 2012). Accordingly, UCB now is considered as the most important novel source for cell therapy. We successfully collect hUCB according to previous mentioned methodology. We isolated, cultured and propagated EPCs. We also can do more EPCs proliferation by endotoxin induced LPS. EPCs were characterized by their ability to culture on fibronectin coated plates and their ability to form EPCs-CFU in culture. Furthermore, EPCs viability was tested by their ability to be counterstained with DAPI (positive blue DAPI-nuclei). Rossella et al. (2009) reported that EPCs do not express procoagulant activity in baseline conditions. However, lipopolysaccharide induces the expression of procoagulant activity. The effect is dose-dependent and reaches statistical significance at 100 ng/mL lipopolysaccharide. On molecular level, we characterized hUCB-EPCs by their high mRNA quantitative expression of VEGFR2/KDR. We also proved high significant expression of VEGFR2/KDR gene in treated EPCs with LPS compared to EPCs alone without LPS. Accordingly, VEGFR2/KDR is one of the most important molecular surface biomarker for EPCs characterization. The essential role of endothelial NO synthase (eNOS) for neovascularization has been described before in several published studies. Aicher et al. (2003) demonstrated that eNOS expressed by bone marrow stromal cells is essential for the mobilization and functional activity of stem and progenitor cells. Thus, low function and insufficient recruitment of EPCs after vascular injury in patients with cardiovascular risk may be due to diminished endothelial NO bioavailability and may lead to aggravation of vascular injury and diminished repair in these patients. Werner et al. (2002) demonstrated that the administration of rosuvastatin to mice with carotid artery injury leads to a delayed beneficial increase of EPC levels that promotes bone marrow-dependent re-endothelialization and diminishes vascular lesion development. Laufs et al. (2004) revealed the effect of physical activity on the production and level of EPCs via an NO-dependent mechanism in mice. These results were confirmed in patients with high vascular risk, that is, individuals with coronary artery disease who were invited to a 4-week training program (Liman and Endres, 2012). Furthermore, all the above demonstrated studies concerning functioning EPCs via eNOS activity and production were coincided with our work. Because we found that hUCB isolated EPCs were expressing eNOS. Furthermore, activated and proliferated EPCs with LPS can express more eNOS and a significant difference between the two studied groups; EPCs and EPCs preconditioned with LPS was found in eNOS expression. Toll-like receptors (TLRs), a kind of pattern recognition receptors (PRRs), mediate the immediate innate immune response and also the subsequent adaptive immune response by activating inflammatory gene transcription and posttranslational processing (Opitz et al., 2009). To date, 12 members of the TLR family have been identified in mammals. Among them, TLR4, recognizing LPS from Gram negative bacteria, can be also activated by viral proteins, endogenous heat shock protein HSP60, oxidized low-density lipoprotein (LDL), and fibrinogen (Akira et al., 2006). TLRs not only initiate and maintain host defenses and inflammation, but also directly involved in the initiation and the progression of diseases such as atherosclerosis. TLR4 overexpression and colocalization have been demonstrated in coronary atherosclerotic plaques (Vasa et al., 2001). Up-regulation of TLR4 causes changes to epithelial cell proliferation, 54 NF k B expression51 and pro-inflammatory cytokine expression55. All three of these are known to be altered during mucositis leading to the suggestion that TLR4 may be a key driver in the pathogenesis of mucositis (Daniel et al., 2013). The activation of TLR4 signaling induces the expression of endothelial cell adhesion molecules, and the production of inflammatory cytokines as well as chemokines, (IL-8, IFN-alpha, IFN-beta, and TNF-alpha) resulting in the increase of neutrophil infiltration, endothelial permeability and intravascular coagulation, which plays important role in the pathophysiology of atherosclerosis (Ye et al., 2008). Therefore, the regulation of TLR4 signaling is important for the prevention and control of inflammatory related diseases. Recently, the effect of extracellular nucleotides on TLR4-mediated immune response has been on controversy. In human monocytes stimulated with LPS, ATP strongly accelerates IL-1β processing and secretion in an autocrine way or by modulating ion influx (Picci et al., 2008).

In dendritic cells, extracellular ATP has been shown to inhibit LPS-induced secretion of the proinflammatory cytokines TNF-α and IL-12, and negatively regulates TLR signaling via P2 receptors (Marteau et al., 2004). He et al. (2010) found that EPCs also express several TLRs, and the activation of TLR4 by LPS induced the phosphorylation of NF-κB, P38, and ERK42/44, and up-regulated the gene expression of cytokines IL-8, IFN-α, IFN-β and TNF-α. These previous several studies coincided with our work regarding TLRs expression and TLR4-mediated signaling induced by LPS in EPCs. We examined hUCB which is the future prospective promise in cell therapy, for EPCs isolation, maintained their culture and propagation. Furthermore, TLR4 was quantitatively expressed by real time PCR in both studied groups; EPCs and EPCs preconditioned with LPS. We report that EPCs and those preconditioned EPCs with LPS isolated from hUCB express EDTLR4 with high significant difference in the later than the former.
Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES


