European Review for Medical and Pharmacological Sciences 2013; 17: 3169-3177

Endothelial progenitor cells are influenced by serum of patients with systemic inflammatory response syndrome or multiple organ dysfunction syndromes

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Abstract. – BACKGROUND AND AIM: To investigate the effects of systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndromes (MODS) on human peripheral blood endothelial progenitor cells.

PATIENTS AND METHODS: Twenty patients admitted to Changhai Hospital, Second Military Medical University, from February, 2011 to November, 2011 were recruited consecutively. The serum samples were collected from the twenty patients who were divided into four groups as following: normal group, post-traumatic group without SIRS, post-traumatic group with SIRS, and post-traumatic group with MODS. Endothelial progenitor cells (EPCs) were isolated from peripheral blood of healthy subjects by using density gradient centrifugation and the effect of the serum on EPCs was detected after stimulating by the serum samples for 0, 6, 12, 24, and 36 h.

RESULTS: Compared with the normal group, the proliferation of EPCs was significantly increased in a time-independent manner in the other three groups, especially in the SIRS serum treated group. The expression of pro-inflammation cytokines was increased in the other three groups compared with the normal group, but the expression of IL-10 in the normal group was higher than the other groups.

CONCLUSIONS: Oxidative stress balance was also broken as the disease progressed. Serum from patients with sepsis could influence proliferation and the inflammation and oxidative stress states of EPCs.

Key Words:
Endothelial progenitor cells, Sepsis, Proliferation, Oxidative stress.

Introduction

Sepsis is a potentially deadly medical condition characterized by a whole-body inflammatory state that is triggered by an infection. It is the major cause of death in post-traumatic patients. Sepsis is a continuous process from asymptomatic to symptomatic infection, systemic inflammatory response syndrome (SIRS), sepsis, and finally multiple organ dysfunctions (MODS). SIRS is a pathologic reaction triggered by a variety of insults including infection, trauma, burns, and acute pancreatitis[1]. It is a serious condition related to systemic inflammation, sepsis, organ dysfunction, and organ failure. SIRS will become more severe when it is complicated by organ dysfunction which cannot be maintained without intervention[2]. Both SIRS and sepsis could ultimately progress to MODS, and eventually death. The mechanisms that lead to death in the setting of sepsis are multifactorial, but alterations in vascular function are one of the primary contributors[3,4]. Endothelial activation is one of the most significant pathophysiologic changes in patients with sepsis. Some previous studies have reported that inflammatory cytokines including TNF-α and LPS could directly activate the receptors on the endothelial surface[5,6]. The damage to the endothelium will cause multi-organ failure, leading to the increased severity and mortality of sepsis.

Endothelial progenitor cells (EPC) are a specific subtype of hematopoietic stem cells that are isolated from circulating mononuclear cells, bone marrow or peripheral blood[7]. They have been identified as circulating cells that counteract ongoing risk factor-induced endothelial cell injury[8,9]. The EPC play a major role in the repair of endothelium, which can migrate to the injured peripheral vascular and differentiate into mature endothelial cells in situ. However, to date, the role of EPC in critical illnesses, especially the relationship of EPC to organ dysfunction or sur-

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vival in sepsis, has not been well explored. In the present study, we aimed to evaluate the effects of serum of patients with SIRS or MODS on the EPCs. EPCs were cultured in a micro-environment mimicking SIRS and MODS, so as to assess the influence of the disease on the cells.

Patients and Methods

Isolation and Culture of EPCs

All persons have given their informed consent prior to their inclusion in the study, and all human studies have been approved by China Ethics Committee and performed in accordance with the ethical standards. Mononuclear cells (MNC) were isolated from human peripheral blood. The MNC were isolated by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare). The preselected EPCs were seeded at a density of 5,000 cells/mm^2 in medium 199, supplemented with 20% FCS (BioWhittaker, Verviers, Belgium), 2 mM L-glutamine, 1% penicillin/streptomycin (both Sigma, St. Louis, MO, USA), 5 U/ml heparin (LeoPharma, Ballerup, Denmark), 10 ng/ml bFGF, 10 ng/ml IGF-1, and 20 ng/ml VEGF (all PeproTech, Rockhill, NJ, USA).

Characterization of EPCs

To verify these culture cells were endothelial progenitor cells, uptake of acetylated LDL, a function associated with endothelial cells, were determined. To detect the uptake of acetylated low-density lipoprotein (Ac-LDL), (DiI-Ac-LDL; cells were incubated with 10 μg/mL Ac-LDL labeled with fluorochrome DiI (Biomedical Technologies, Stoughton, MA, USA) for 4 h. Cells were then fixed with 4% paraformaldehyde and incubated with 10 μg/mL FITC-labeled Ulex europaeus agglutinin-I (lectin; Sigma) for 1 h. DiI-Ac-LDL/lectin double-positive cells were identified as EPCs. Three different fields of cells were counted under the confocal microscope with a magnification of 200×. The flow cytometry analysis of surface markers including CD34, CD133, CD45, and KDR, was also carried out as published previously.

Angiogenesis Assay

An *In Vitro* Angiogenesis Assay Kit (ECM625, Chemicon) was used to assess the angiogenetic capabilities of EPCs. The 96-well plate coated with Matrigel Matrix was incubated at 4°C for 24 h and then cultured at the incubator for 30 min. The EPCs were added to the wells at a density of 4×10^5/well. The angiogenesis assay plate was incubated for 16-18 h at 37°C, 5% cabon dioxide. Following the incubation, the medium was carefully removed from the plates. The plates were washed twice by adding 100 l HBSS to each well, and then the EPCs were labeled by adding add 50 L/well of (8 g/mL) Calcein. After incubating for 1 h at 37°C, the labeling solution was removed. The tubes in the plate were counted by using a fluorescent microscope.

Serum Collection and Cell Treatment

The serum was collected from twenty patients who were admitted to Changhai Hospital, Second Military Medical University, from February to November in 2011. The patients were divided with four groups as following: Normal, WS group (post-traumatic without SIRS), Post-traumatic with SIRS, and post-traumatic with MODS. The serum was collected from the post-traumatic patients having major surgery, burns, post-traumatic within 24 h (not treated with anti-oxidants). Patients who meets ≥ 2 of the following criteria were identified as the patients with SIRS: (1) Temp > 38°C or < 36°C, (2) heart rate > 90/min, (3) respiratory rate > 20/min, (4) WBC > 12×10^9/L or < 4×10^9/L. While, the SIRS patients with more than one of the symptom including ARF, ARDS, AHF, DIC, shock, and acute brain failure were recognized as the MODS patients.

The central venous (5 mL) or peripheral venous blood (10 mL) was collected from each selected patients. The coagulated blood was centrifugated at 3400 r/min for 30 min at 4°C, and then, the upper serum was transferred into the 1.5 ml centrifuge tubes.

The human peripheral blood endothelial progenitor cells were cultured with 10% medium 199 supplemented with the collected serum for different time points (6, 12, 24, and 36 h).

Cell Proliferation

The EPCs were added to the 96-well plate at a density of 1×10^4 /well, and were incubated at 37°C, 5% cabon dioxide. Then, 10 l CCK-8 was added to each well. After incubating at 37°C, 5% cabon dioxide for 1-4 h, the absorbance for all the 96-well plates were determined at 450 nm using the Microplate Reader.

Real-time PCR

Total mRNA of EPCs was extracted using Trizol reagent (Invitrogen) according to the manufac-

turers' instructions. With random hexamer primers, the maximum allowed volumes of mRNA samples were converted to cDNA by using the ExScript RT reagent kit (TaKaRa). The mRNA sample was used as the negative control. Primers and probes for human HO-1, NADPH p22phox, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were designed byPrimer Express 2.0 software (Applied Biosystems) and synthesized by Genecore. The sequences of the primers are shown in Table I. The PCR amplification cycle was set as 95°C for 10 s for initial denaturation, and then performed for 55 cycles under the following conditions: 5 s at 95°C, and 20 s at 60°C. Copy numbers were obtained through the extrapolation of Ct values of the test samples against the corresponding standard curve, and the housekeeping gene GAPDH was used to normalize the amount of mRNA from each sample.

Migration Assay

Isolated EPCs were detached using 1 mmol/l ethylenediamine tetraacetic acid in PBS (PH 7.4), were harvested by centrifugation, were resuspended in M-199 medium and counted. EPCs (1×10^4) were added to the upper chamber of a modified Boyden chamber with a polycarbonate filter (6.5 mm diameter, 8 μm pore size; Neuro Probe, Gaithersberg, MD, USA), and 500 μL M-199 with human recombinant vascular endothelial growth factor (VEGF) (50 ng/ml) (Peprotech EC, London, UK) was added to the bottom chamber. After 24 h of incubation at 37°C and 5% carbon dioxide, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification of migrated cells, cell nuclei were stained with Giemsa (Dade Behring, Marburg, Germany). Migrated cells in the lower chamber were counted manually in five random microscopic fields.

Table I. Baseline characteristics of the patients included in the study.

Primer	5′-3′
HO-1-F	AGGAGGAGATTGAGCACAAC
HO-1-R	CGTACCAGAAGGCCATGTC
NADPH F p22phox-	ATCAGGGTGCAGAGGTGTTC
NADPH p22phox-R	AAACACTGACAAGTGCCCCT
GAPDH-F	ACACTGTGCCCATCTACGAGGGG
GAPDH-R	ATGATGGAGTTGAAGGTAGTTTCGTGGAT

Measurement of Expression of Inflammatory Cytokines

Levels of MMP-9, MCP-1, IL-8, and IL-10 were detected by ELISA kits according to the manufacturer's instructions.

Statistical Analysis

All the data analysis was carried out using the statistical analysis software SPSS 11.5 for windows (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD) with the range given in parentheses. Significant differences between the groups were analyzed using paired Student's *t*-test, ANOVA, and linear regression when the data were normally distributed. The Pearson's m2 test was carried out to analyze the differences between two or more groups. The differences were considered significant at $p < 0.05$.

Results

The Morphology of Endothelial Progenitor cell Colonies

Blood samples were drawn from peripheral veins and MNCs were isolated by density gradient centrifugation. The mononuclear cells separated were round and small. The non-adherent cells were removed after 24 h (Figure 1A). After 7 days of culture, adherent cells formed some colonies, and some cells gradually turned into spindle cells (Figure 1B). Fourteen days later, lots of cobblestone-like cells were appearing among the spindle cells (Figure 1D).

Characterization of Endothelial Progenitor Cells

The attached spindle cells at different time pointes were all positive for taking up Dil-ac-LDL and UEA-1. Co-staining cells revealed that about 80% of adherent cells were both Dil-ac-LDL-positive and UEA-1-positive (Figure 2).

Angiogenesis Test

The number of tubes in the plate was used to illustrate the angiogenesis function of EPCs at different time points. The results showed that EPCs could form vascular networks and channels after 7 days of culture *in vitro* (Figure 3).

The cell Proliferation of EPCs After Stimulating by the Serum of Patients

Compared with the EPCs stimulated by the serum from normal subjects, cells treated by the

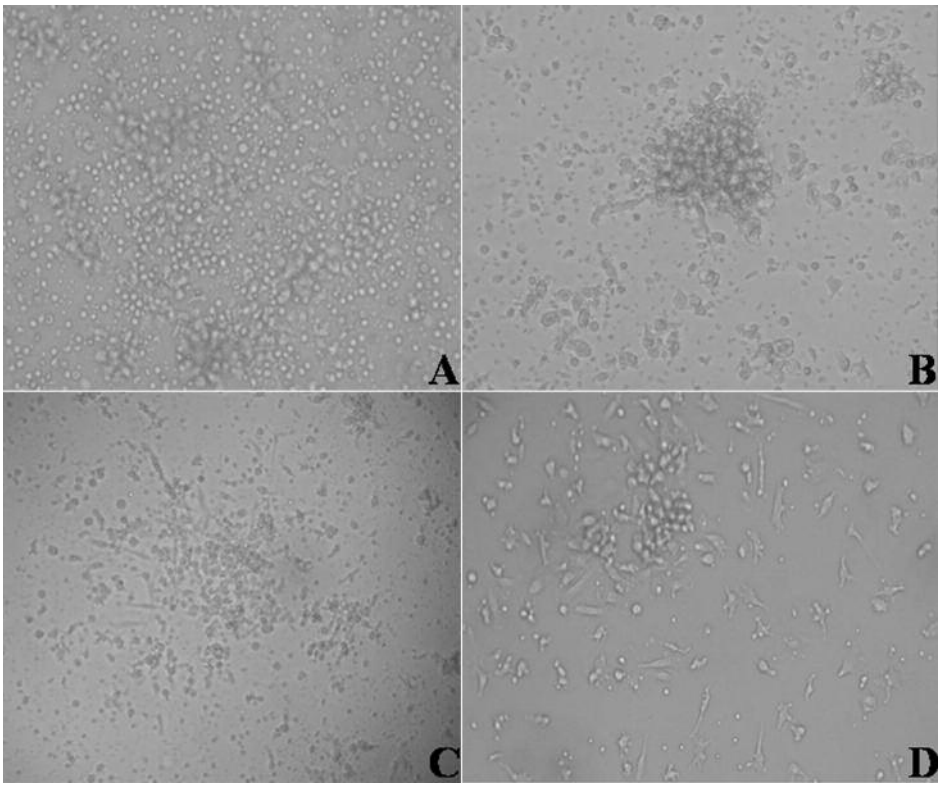

Figure 1. Morphological changes of endothelial progenitor cells are at different time points. The mononuclear cells separated were round and small. The nonadherent cells were removed after 24 h. After 7 days of culture, adherent cells formed some colonies, and some cells gradually turned into spindle cells. 14 days later, lots of cobblestone-like cells were appearing among the spindle cells. A: The second day; B: The sixth day; C: The eighth day; D: The fourteenth day.

other serum grew much faster, even as early as 6 h after the stimulation. The proliferation of EPCs were significantly increased in a time-independently manner. The cell number was increased in a time-dependent manner in the normal serum treated cells, while, no significant differences were observed among the 6, 12, 24, and 36 h stimulated group. Cell number of SIRS serum-

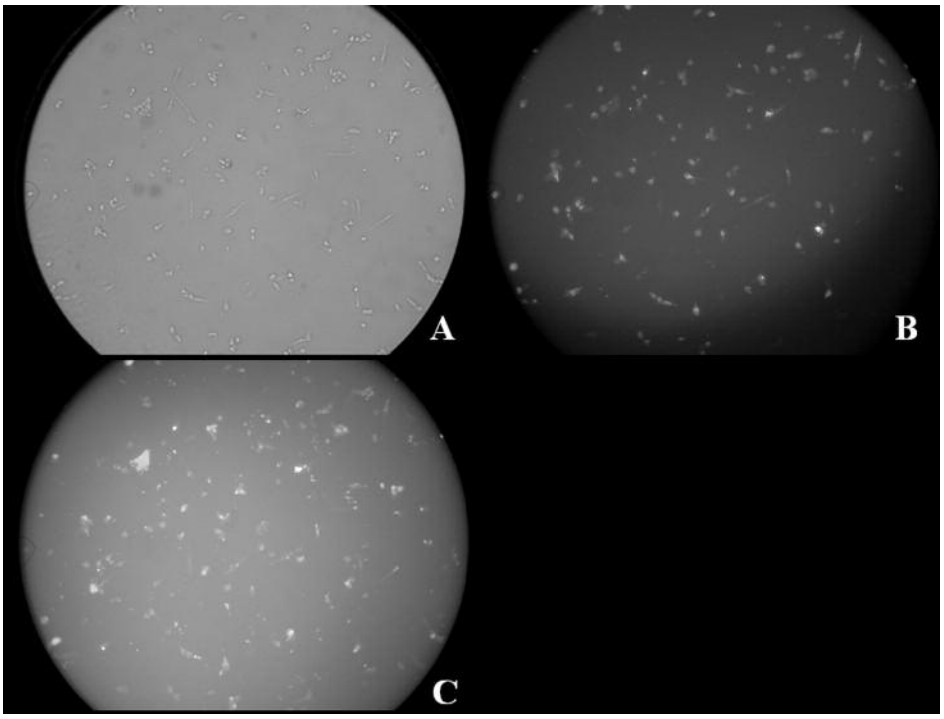

Figure 2. The ability of endothelial progenitor cells uptaking DiI-Ac-LDL and UEA. The attached spindle cells at different time pointes were all positive for taking up Dil-ac-LDL and UEA-1. Co-staining cells revealed that about 80% of adherent cells were both Dil-ac-LDL-positive and UEA-1-positive A: the general shape of EPCs; B: EPCs uptaking of DiI-Ac-LDL; C: EPCs binding with FITC-UEA-I.

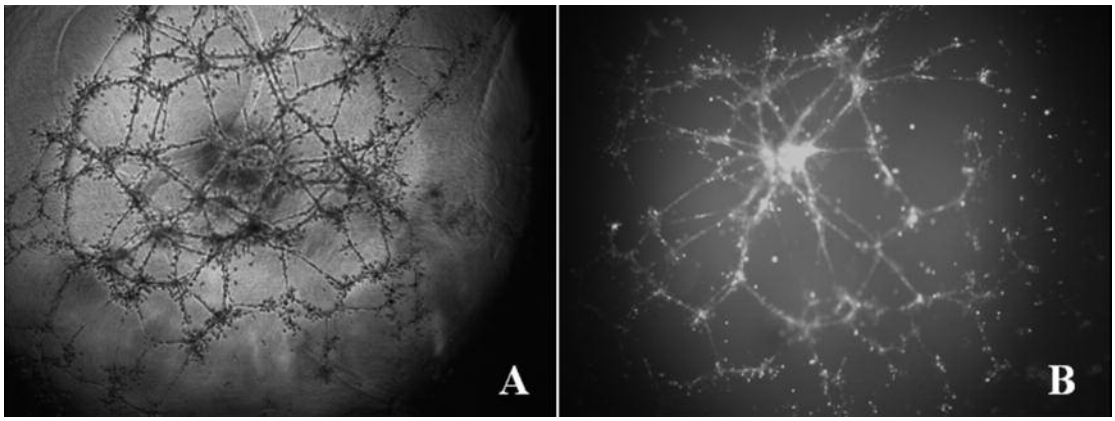

Figure 3. Angiogenesis function of endothelial progenitor cells. Angiogenesis function of endothelial progenitor cells with Calcein added, observed by ***(A)*** phase-contrast microscope and ***(B)*** fluorescent microscope. Magnification: ×400.

treated EPCs was significantly increased compared with other stimulated group at any time point ($p < 0.05$) (Figure 4).

Migratory Capacities of Endothelial Progenitor Cells

In this study, the migratory capacities of EPCs in peripheral circulation was gradually enhanced in WS group (1.25±0.10) and SIRS serum-treated group (1.90±0.15), but was impaired in MODS serum-treated group (0.87±0.15). These data indicated that the migratory capacities of EPCs were impaired after the MODS serum stimulation.

Levels of VEGF and Cytokines in Endothelial Progenitor Cells

Compared with the normal group (84.64±9.56 pg/ml), the levels of VEGF were significantly increased ($p < 0.01$) in WS group (132.72±13.33

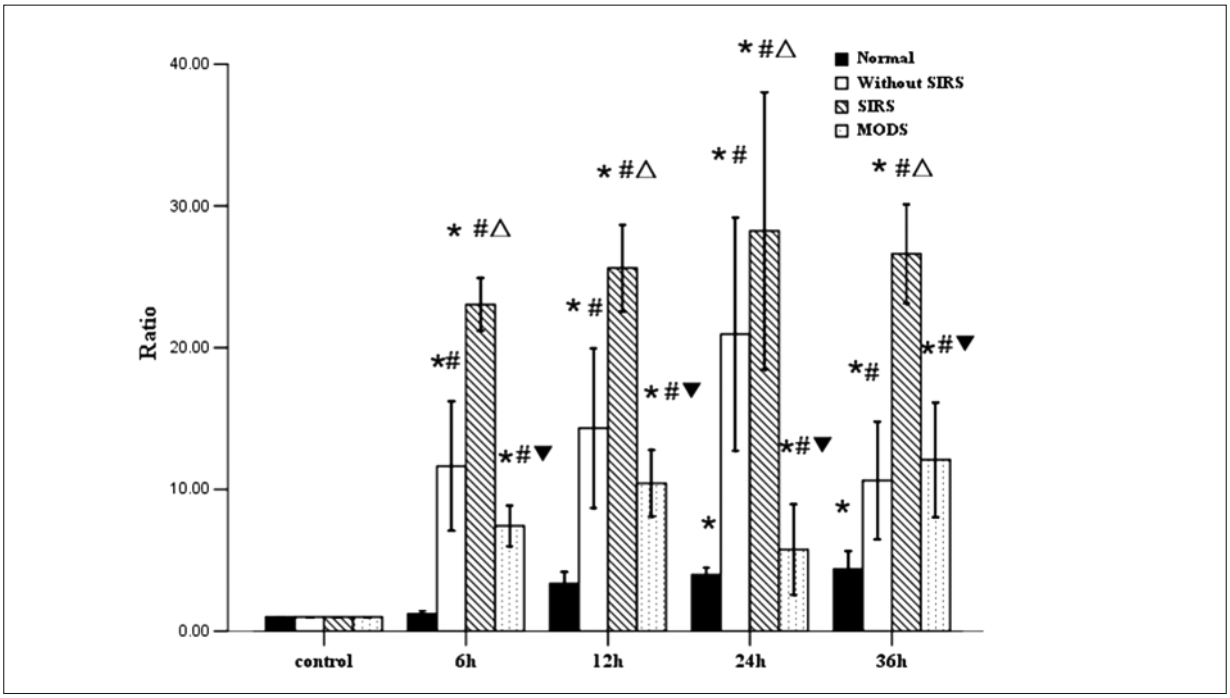

Figure 4. The cell proliferation of endothelial progenitor cells at different time points. *Compared with the control group, $p < 0.05$; #Compared with the normal group at the same time, $p < 0.05$; ΔCompared with the without SIRS group at the same time, $p < 0.05$; †Compared with the SIRS group at the same time, $p < 0.05$

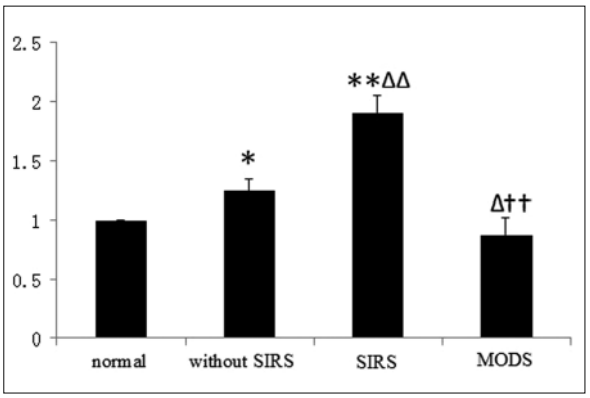

Figure 5. The migratory abilities of endothelial progenitor cells treated with patient's serum. *Compared with the normal group, $p < 0.05$; **Compared with the normal group, $p < 0.01$; $^{\Delta}$Compared with the without SIRS group, $p < 0.05$; $^{\Delta\Delta}$Compared with the without SIRS group, $p < 0.01$; ††Compared with the SIRS group, $p < 0.01$.

pg/ml) and the SIRS serum-treated group (219.48±22.81 pg/ml) (Figure 6). However, in MODS serum-treated group, the level of VEGF (153.36±11.40 pg/ml) was significantly decreased ($p < 0.01$) compared with that of the EPCs treated with SIRS serum (Figure 5), indicating that the angiogenesis ability of EPCs was impaired. We observed that MCP-1 levels were also significantly increased ($p < 0.05$) in a similar manner to the levels of VEGF, and levels of MCP-1 in the normal group, WS group, the SIRS serum-treated group, and the MODS serum-treated group were 139.35±14.06 pg/ml, 173.66±5.94 pg/ml, 185.72±8.15 pg/ml, and 149.86±8.34 pg/ml, respectively (Figure 6). Levels of MMP-9 in SIRS (87.52±4.33 pg/ml) and MODS serum-treated groups (96.13±5.5 pg/ml) were significantly increased ($p < 0.05$) compared with the normal group (79.63±4.62 pg/ml), and signifi-

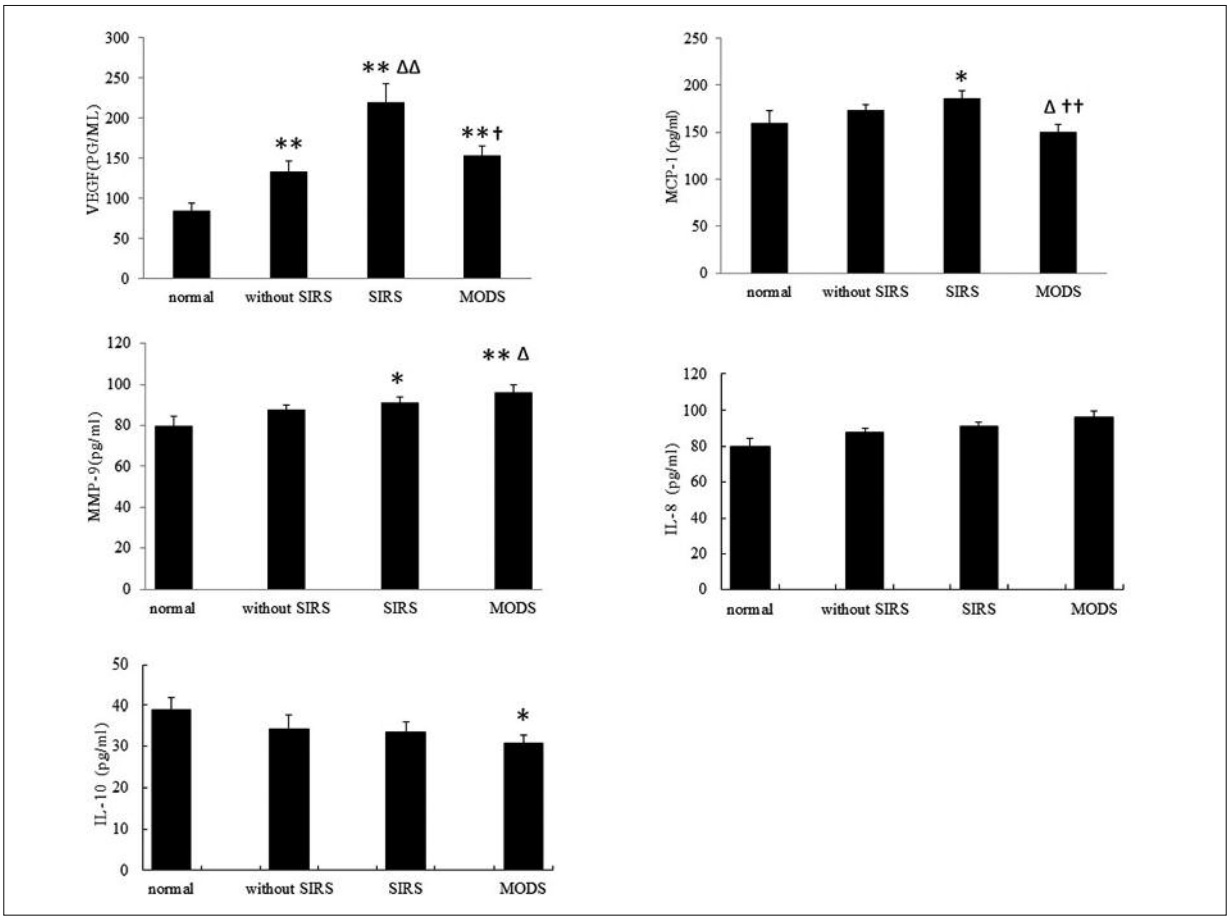

Figure 6. The levels of VEGF pro-inflammatory cytokines in endothelial progenitor cells treated with patient's serum. *Compared with the normal group, $p < 0.05$; **Ccompared with the normal group, $p < 0.01$; $^{\Delta}$Compared with the without SIRS group, $p < 0.05$; $^{\Delta\Delta}$Compared with the without SIRS group, $p < 0.01$; †Compared with the SIRS group, $p < 0.05$; ††Compared with the SIRS group, $p < 0.01$.

cant difference was also observed between the SIRS and MODS serum-treated group ($p < 0.05$) (Figure 6). Expression of IL-8 also increased as the disease progressed, but no statistical difference between the normal group and the other three groups were observed (Figure 6). However, compared with the normal group (38.94±3.01 pg/ml), the levels of IL-10 were significantly decreased in the MODS serum-treated group ($p < 0.05$) (30.71±2.00 pg/ml) (Figure 6).

Levels of HO-1 and p22phox mRNA in Endothelial Progenitor Cells

The levels of HO-1 (1.18±0.21) and p22phox (1.35±0.19) were significantly increased at 12 hour of SIRS serum treatment ($p < 0.05$). No significant difference of p22phox level was observed between the normal group and the MODS serum treated group. However, compared with the normal group, level of HO-1 was remarkably decreased ($p < 0.05$) (0.87±0.27) (Figure 7)

Discussion

In this research, we report that the function of EPCs would change in different process of sepsis. The cell proliferation and the migration abilities of EPCs in the SIRS serum treated group are enhanced compared with the normal and WS group. However, if the sepsis progresses into MODS, the function of EPCs would significantly decline. Related genes of oxidative stress are positively correlated with the stage of sepsis. These data are consistent with the previous study[10,11].

Of the mechanisms that lead to the sepsis-related organ dysfunction and death, endothelial dysfunction is one of the primary contributors.

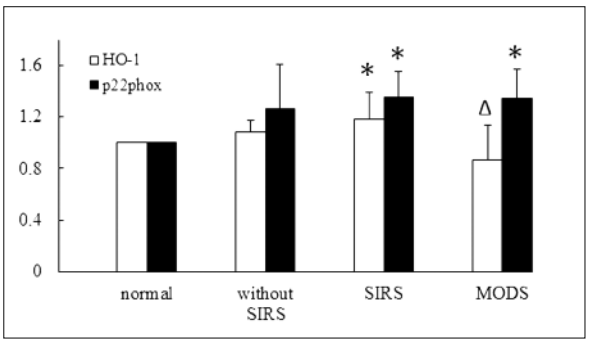

Figure 7. Levels of HO-1and p22phox mRNA in endothelial progenitor cells cultured with serum from patients. *Compared with the normal group, $p < 0.05$; ΔCompared with the without SIRS group, $p < 0.05$.

It is generally accepted that endothelial progenitor cells play an important role in the repair process. Clinical researchers have demonstrated that the number of EPCs is directly correlated with the clinical prognosis of patients with sepsis or levels of pro-inflammatory cytokines in serum of patients with sepsis[3,12]. Beck et al[12] showed that the number of EPCs in septic patients was significantly higher than that in the control population and was correlated with survival of patients and the levels of cytokines. These results are consistent with our study, indicating that existence of dysfunction of endothelial progenitor cells in sepsis. In the present work, compared with the serum in normal people, the cell proliferations of EPCs treated with the serum sample were significantly enhanced peaked at the 6 hour treatment of serum in patients with sepsis. Rafat et al[13] reported that the circulation endothelial progenitor cells (CEPCs) increased from the 6th hour after diagnosis as sepsis and remained high during the sepsis phase. These findings were in accordance with our reports. However, the specific mechanism of this action still remains to be determined.

As we know, the functions of EPCs *in vitro* would remain stable after short-time culturation[14]. In this study, in order to investigate the functions of EPCs, the serum of patients with sepsis was used to stimulate the EPCs. The cell proliferation of EPCs were remarkably enhanced by the SIRS serum treatment, compared with that of the post-traumatic without SIRS or the post-traumatic with MODS treated EPCs. Not only that, the expression of inflammation cytokines (such as IL-4, IL-10, and TGF- 1) or toxin are reduced in patients with SIRS or MODS, and finally decreasing the number of EPCs in the MODS serum treated group. These observations may result from the higher levels of inflammatory cytokines including IL-1, TNF, and IL-8, which are the major contributing factors to cell proliferation of EPCs, in SIRS serum treated group. Beck et al[12] reported that the number of EPCs is significantly increased in septic patients, and was correlated with the survival of patients and the concentration of cytokines. These data were consistent with our results. The increased number of EPCs found in this study might therefore be a consequence of the body's attempting to limit vascular damage by inducing endogenous endothelial repair mechanisms[15-17].

In thisinvestigation, we found that the number of EPCs stimulated by the serum from the nor-

mal patients was increased in a time-dependent manner, however, no similar results were observed in the other three groups. These may result from the increased expression of proflammatory cytokines, toxins or increasing generation of reactive oxygen species (ROS) in the serum from the patients with sepsis[18-20]. Therefore, even though the EPCs can be stimulated to proliferate rapidly by the serum of the patients with sepsis, the cell proliferation of the EPCs will be inhibited by the interaction of other molecular including ROS or toxins after 24 hours treatment.

ROS is an important mediator in different signaling pathways of angiogenesis and cell metabolism modulating proliferation, migration and angiogenesis in mature endothelial cells[21]. Excessive production of ROS would induce severe damages to DNA and proteins, and eventually leading to endothelial dysfunction and cell apoptosis[22,23]. It has been confirmed that NADPH oxidase is a major contribution to vascular oxidative stress, and p22phox subunit may also be a critical component of ROS-generating vascular damage. Due to its phenolic structure, heme oxygenase-1 (HO-1) can act as an antioxidant[24]. In this study, the expression of HO-1mRNA and the inflammatory cytokines was significantly increased in the EPCs treated with the serum from the patients with SIRS, however, was significantly reduced in EPCs treated with the MODS serum. It is suggested that when sepsis progresses to MODS, the compensatory effects of anti-oxidant enzyme system was severely damaged, thus leading to and could oxidative stress ineffective resistance. In addition, level of p22phox subunit mRNA was increased with the severity of the infection, finally leading to the excessive oxidative stress. Our research showed that oxidative stress could induce dysfunction of EPCs by inhibiting their mobilization, proliferation, and migration, thus leading to the acceleration of cell aging and apoptosis. So the EPCs-dependent vascular repair could be enhanced by improving the resistance of EPCs to oxidative stress, and finally offering better therapy for patients with sepsis.

Conclusions

The numbers and functions of EPCs are changed with the increased level of inflammation. This may be due to the interaction among a variety of cytokines and ROS. Our paper may provide more evidence for the clinical implications in treatment of patients with sepsis. However, further prospective studies are needed to carry out on how to slow down or control the progress of MODS by maintaining the number of EPCs in patients with antioxidants.

Conflict of Interest

The Authors have no financial conflicts of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81071543/H1504, 81101418/H1504)

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