Original Paper

Effect of Lipopolysaccharide Mediating Early- and Late- Activated THP-1 Macrophages on ECV304 Endothelial Cell Dysfunction: Dysregulation of Secretion of VEGF and Proliferation and Migration of ECV304

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Key Words

Macrophages • ECV304 • VEGF • Proliferation • Migration

Abstract

Objective: To investigate the different effects of lipopolysaccharide (LPS) mediating early and late activated THP-1 macrophages (Mφ) on ECV304 endothelial cell dysfunction: dysregulation of secretion of VEGF and proliferation, and migration of ECV304. **Methods:** The inflammatory Mφ was divided into early phase (2 h) group and late phase (24 h) group according the different exposure time to LPS. Then the inflammatory Mφ and ECV304 were co-cultured via transwell chambers in both non-contacting and contacting systems. The levels of VEGF were determined by ELISA, and the proliferation index and apoptosis of ECV304 were analyzed by FACSCalibur. The migration of ECV304 was tested by modified Boyden chamber assay. **Results:** The level of VEGF and the proliferation of ECV304 cells were increased more apparently in early-phase Mφ-treated group. But the proportion of early apoptotic and late apoptotic/necrotic cells in late-phase Mφ-treated group were higher than that of the former. Migration rate of ECV304 was enhanced in early-phase Mφ-treated group. All those effects were more significant in contacting system comparing with no-contacting system. **Conclusion:** Early-activated macrophages (mediated by LPS) could increase the secretion of VEGF and promote the proliferation and migration of ECV304; while the late-activated macrophages could promote/enhance the apoptosis of ECV304 more significant in contacting system when (it was) compared with no-contacting system.

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Introduction

The endothelium is a kind of highly dynamic cell layer involving in a multitude of physiological functions, including vascular tone control, movement of cells and nutrients, maintenance of blood fluidity and growth of new vessels [1]. Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease and was considered as the first step toward coronary arteriosclerosis [2-4]. The macrophages (M ϕ) related inflammatory response was associated with both systemic inflammation and endothelial dysfunction in the carotid arteries and diabetic patients [5, 6]? It is of great interest that M ϕ can support growth of micro-vascular endothelial cells (EC) when both kinds of cells were co-cultured [7]. Recent studies have shown that the expressions of many genes encoding transcription factor, cytokines, cell signaling modulators and apoptosis associated proteins were changed in the M ϕ which was activated by lipopolysaccharide (LPS) in early and late phase [8-10]. And the migration of M ϕ was also different between early and late phase [11]. However, the effect of LPS mediating early and late activated M ϕ on endothelial cell dysfunction is unknown. So in this work the effects of early and late M ϕ (THP-1) which was activated by LPS on secretion, proliferation and migration of ECV304 were investigated. Although ECV304 line is commonly thought to be a derivative of a human urinary bladder carcinoma T24 (epithelial) line, does also show endothelial characters. When in the absence of an ideal model, ECV304 cells remain thought to be useful in studies of endothelial functions [12]. Epithelial cell co-culture models have recently been extended to primary cells to define epithelial cell function in immune reactivity to microorganisms. Many diverse, sometimes intricate, epithelial cell co-culture models based on the use of single lineages of cells, also known as monocultures, have been characterized under carefully optimized *in vitro* conditions [13].

Materials and Methods

Cell lines and cell culture

The human monocytic cell line THP-1 cells (kind gift from Prof. SL Huang) and ECV304 cells (obtained from Cell Bank, Center of Experimental Animals, Sun Yat-Sen University, Guangzhou, China) were cultured according to the supplier's recommendations. ECV304 cells appear to be a valuable model for the study of cellular processes in the endothelium, including cell-cell adhesions, angiogenesis, and signal transduction [12]. THP-1 monocytes were cultured in RPMI 1640 medium, and ECV304 cells were cultured in high glucose DMEM. Each medium contained 10% fetal bovine serum (all from Gibco, UK), 100 units/mL penicillin/streptomycin, and 2 mM L-glutamine. The medium was renewed every 48 h. Cells were cultured at 37°C and in CO₂ air atmosphere with a humidity of 5% (v/v).

Differentiation and activation of THP-1 cells

Phorbol 12-myristate 13-acetate (PMA) (Sigma) was used to stimulate monocyte cells to differentiate into M ϕ . In contacting study, transwell chambers with a 0.4 μ m-pore-size membrane was used and 1×10^6 THP-1 cells were seeded into upper chamber of a six-well transwell and treated with 320 nM PMA. In no-contacting study, membrane with the same pore size was used and 1×10^6 THP-1 cells were seeded into the bottom of lower chamber. After incubation for 24 hours, and when cells showed macrophage characteristics under microscope, nonattached cells were removed by aspiration, and the adherent cells were washed with RPMI 1640 three times to remove all PMA [14]. Then the PMA-treated THP-1 macrophages were stimulated by LPS (1 μ g/mL, Sigma, USA) for 2 h or 24 h.

Co-culture of M ϕ and ECV304 cells

After being removed all LPS in contacting study, ECV304 cells (1×10^6) were cultured on the reverse side of the membrane. In no-contacting study, 1×10^6 ECV304 cells were seeded into the upper chamber. All the media was lack of serum and growth factors. After co-cultured for 24 h, the ECV304 cells and the supernatant of upper chamber were collected for subsequent detection (Fig. 1 Contacting and Non-contacting).

Fig. 1. Experiment arrangements. In contacting study (pore size 0.4 μm), Mφ (2h or 24h) were seeded on one side of the membrane and ECV304 cells were cultured on the reverse side of the membrane. In no-contacting study (pore size 0.4 μm), Mφ (2h or 24h) were seeded into the upper chamber and ECV304 cells were seeded into the upper chamber. Boyden chamber assay (pore size 8 μm) was performed under the above conditions to analyze the effect of Mφ (2h or 24h) on ECV304 cell migration.

Measurement of VEGF

Level of vascular endothelial growth factor (VEGF) was measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA). The absorbance was read at 450nm using a Spectra Max 340 microplate reader (Bio-Tek Instruments, USA). In order to eliminate any possible effect of Mφ, the effect of Mφ on the secretion of ECV304 was approximate calculated using the formula i.e. VEGF of ECV304 = co-cultured ECV304 and Mφ group – individual Mφ cultured (control group).

Determination of proliferation index

Cell cycle fractions were determined by propidium iodide nuclear staining. Briefly, 1×10^6 cells were harvested and washed in PBS, then were fixed with 70% ethanol, and incubated with PI for 30 minutes at 37°C. Data were collected and analyzed by FACSCalibur. Cell cycle analysis was done by applying diploid cell cycle model using the ModFit LT (Verity Software House, ME). Percentage of cells in each cell cycle phase (G0/G1, S and G2/M), and the proliferation index were calculated through the proliferation index formula $[(S+G2/M)/(G0/G1+S+G2/M) \times 100\%]$.

Determination of apoptosis

The proportion of necrotic and apoptotic after induction of cell death were assessed by annexin V-FITC staining using a FCM apoptosis detection kit (BD Biosciences, USA). 1×10^6 cells were harvested and washed with binding buffer, and then resuspended in 100 μl binding buffer containing 10 μl annexin V-FITC. After 15 min of incubation at room temperature in the dark, the cells were washed with the binding buffer. Just before analysis of the samples by a flow cytometry (FACSCalibur, Becton Dickinson, USA), propidium iodide (PI) was added (final concentration 5 μg/ml) to distinguish early apoptotic cells (annexin V+/PI-) from late apoptotic/ necrotic cells (annexin V+/PI+). The samples were analyzed through a FACSCalibur (Becton Dickinson) instrument installed CellQuest software.

Modified Boyden chamber assay

Modified Boyden chamber assay (Pore size 8 μm, Costar Corp. USA) was performed under the above conditions to analyze the effect of Mφ on ECV304 cell migration (Fig. 1. Boyden chamber assay). Cell numbers in the downside and upside of the membrane were determined using the colorimetric crystal violet (CV) assay. Cells were stained with 0.5% CV (in 20% methanol; Sigma) for 10 min at room temperature. After that, cell-bound dye was dissolved in citrate-buffered ethanol (0.05 M in 50% ethanol) and quantified at 550 nm in Spectra Max 340 microplate reader (Bio-Tek Instruments, USA). Each treatment was measured in triplicate of two independent Experiments and the mean of six counts was used as the value of migrated cell number. The cell migration rate was calculated $[= \text{number of cells permeating through the membrane (number of cells in the downside of membrane)} / \text{total number of cells (number of cells in the upside of membrane} + \text{number of cells in the downside of membrane)} \times 100\%]$.

Fig. 2. Effect of lipopolysaccharide mediating early and late-activated THP-1 macrophages on the VEGF secretion of ECV304. In contacting co-culture system, the level of VEGF in E M ϕ -treated group was significantly higher than that in L M ϕ -treated group. In non-contacting co-culture system, the level of VEGF was also increased in E M ϕ -treated group compared with L M ϕ -treated group. But the increase of VEGF was more pronounced in contacting co-culture system. **: $P < 0.01$.

Fig. 3. Effect of lipopolysaccharide mediating early and late-activated THP-1 macrophages on the proliferation index of ECV304. In contacting co-culture system, the proliferation index (PI) in E M ϕ -treated group was increased significantly higher than that in L M ϕ -treated group. In non-contacting co-culture system, the PI increased more pronounced in E M ϕ -treated group than that in L M ϕ -treated group. And the increase of PI was more pronounced in contacting co-culture system. **: $P < 0.01$.

Statistical analyses

All data were expressed as the mean $\pm$ standard error of the mean (SEM). Student-t-test was applied to compare means of two independent samples. Each measurement was performed at least in triplicate. One-way ANOVA followed by the Dunnett or Duncan test for multiple comparisons, as appropriate. Differences were considered statistically significant when $P < 0.05$. All statistical analysis was performed with SPSS statistical software (version 13.0 for Windows).

Results

Effect of lipopolysaccharide mediating early and late activated THP-1 macrophages on the secretion of VEGF of ECV304

In contacting co-culture system, the level of VEGF in E M ϕ treated group was significantly higher than that in L M ϕ treated group (978.3 ± 50.4 vs. 825.1 ± 29.7 , $P < 0.01$). Also the level of VEGF in L M ϕ -treated group was increased compared with control group. In non-contacting co-culture system, the level of VEGF was also increased in E M ϕ treated group compared with L M ϕ treated group (857.6 ± 39.4 vs. 725.5 ± 28.5 , $P < 0.01$). However, the increase of VEGF was more significant in contacting co-culture system (E M ϕ -treated group VEGF, non-contacting vs. contacting: 857.6 ± 39.4 vs. 978.3 ± 50.4 , $P < 0.01$; L M ϕ treated group VEGF, non-contacting vs. contacting: 725.5 ± 28.5 vs. 825.1 ± 29.7 , $P < 0.01$) (Fig. 2).

Effect of lipopolysaccharide mediating early and late activated THP-1 macrophages on the proliferation of ECV304

The proportions of ECV304 nuclei in the S plus G2/M fractions were compared between E M ϕ -treated group and L M ϕ -treated group. In contacting co-culture system, the proliferation index (PI) in E M ϕ -treated group was increased markedly higher than that in L M ϕ -treated group (53.15 ± 8.19 vs. 42.41 ± 8.30 , $P < 0.01$). And also in non-contacting co-culture system, the PI increased more apparently in E M ϕ -treated group than that in L M ϕ -treated group (46.41 ± 7.63 vs. 37.85 ± 6.69 , $P < 0.01$). However, the increase of PI was more significant in contacting co-culture system (E M ϕ -treated group PI, non-contacting vs. contacting: 46.41 ± 7.63 vs. 53.15 ± 8.19 , $P < 0.01$; L M ϕ -treated group PI, non-contacting vs. contacting: 37.85 ± 6.69 vs. 42.41 ± 8.30 , $P < 0.01$) (Fig. 3).

Fig. 4. Effect of lipopolysaccharide mediating early and late-activated THP-1 macrophages on the apoptosis of ECV304. The apoptotic cell was assessed by measuring the phosphatidylserine residues on the cell surface as described in the “Material and Methods” (double staining with annexinV/FITC and PI). Both in non-contacting and contacting co-culture systems, the proportion of early apoptotic, late apoptotic/necrotic cells in L Mφ-treated group were increased pronounced than that in E Mφ-treated group. And this trend was more significant in contacting co-culture system. **: $P < 0.01$.

Fig. 5. Effect of lipopolysaccharide mediating early and late-activated THP-1 macrophages on the migration of ECV304. E Mφ and L Mφ were both able to stimulate the migration of ECV304. But the migration rate in E Mφ-treated group was more pronounced than that in L Mφ-treated group. **: $P < 0.01$.

Effect of lipopolysaccharide mediating early and late activated THP-1 macrophages on apoptosis of ECV304

Precise cytometric detection and quantitation of cells undergoing programmed cell death (known as apoptosis) or cells dying from necrosis were determined by annexin V-FITC and propidium iodide staining and analyzed by FCM. Both in non-contacting and contacting co-culture systems, the proportion of early apoptotic and late apoptotic/necrotic cells were increased significantly in L Mφ-treated group compared with that in E Mφ-treated group. And this trend was more significant in contacting co-culture system (Fig. 4).

Effect of lipopolysaccharide mediating early and late activated THP-1 macrophages on the migration of ECV304

The motility was evaluated by modified Boyden chamber assay. These tests demonstrated that E Mφ and L Mφ were both able to stimulate the migration of ECV304. But the migration rate in E Mφ-treated group was more significant than that in L M- treated group (14.67 ± 0.49 vs. 8.33 ± 0.46 , $P < 0.01$) (Fig. 5).

Discussion

In present study, transwell model, which was useful for the study of cell-cell communication, was applied to provide evidences for that LPS mediating early and late activated Mφ had various effects on endothelial cell dysfunction [15]. Intercellular communication can be roughly divided into two types, i.e. direct interaction between membrane molecules of two adjacent cells (cell-cell contact) and indirect interaction with

secretion of diffusible factors (cell-cell no-contact) which can activate specific receptors on the target cells [16, 17]. The transwell co-culture system described herein allows for cell-cell communication with soluble factors yet no direct cell-cell contact, which can simulate *in vivo* cartilaginous environment [18]. To elucidate the mechanism of interaction between human retinal progenitor cells and human umbilical vein endothelial cells, non-contacting and contacting co-culture transwell (pore size 0.4 μ m) cell culture systems were developed [19]. In this study, non-contacting and contacting co-culture transwell cell culture systems were used and a pore size of 0.4 μ m was recommended. In non-contacting co-culture system, mediators secreted by M ϕ cells can lead to secretion, migration, proliferation, apoptosis and necrosis of ECV304. In contacting co-culture system, the M ϕ cells had effect on the function of ECV304 by vesicles derived from membrane.

Phorbol 12-myristate 13-acetate (PMA) (Sigma) was used to stimulate monocyte cells to differentiate M ϕ . Prior methods of monocyte maturation have used 72h of treatment with PMA [20]; in our study 24h was chosen because it was found already sufficient to induce maturation. M ϕ activated by LPS in early and late phases have different biological functions. LPS-induced M ϕ model is a useful protocol *in vitro* screening to identify new anti-inflammatory compounds [21]. A previous study suggested that tumour necrosis factor α (TNF- α) is a key pro-inflammatory cytokine when the M ϕ was activated by LPS [22]. And also the morphological changes taking place in macrophages during the activation. The results show that late-activated M ϕ extended more pseudopodia than early-activated M ϕ (data not shown). Then the different effects on endothelial cell of early or late activated M ϕ were studied in subsequent study.

As a potent mitogen, VEGF is a permeability factor which associates with the growth and migration of endothelial cell [23, 24]. LPS could significantly enhance the secretions of VEGF of endothelial cells (Ecs) co-cultured with macrophages, whereas no such effect was observed in single-cultured Ecs [25]. In our study we found that both the early and late activated M ϕ could increase the secretion of VEGF of ECV304 in both contacting and no-contacting co-culture systems, and yet the influences were more significant in early Activated M ϕ treatment group and in contacting system. These results were consistent with previous research in which was found that the proliferation of endothelial cell induced by the peritoneal fluid macrophages in endometriosis should attribute to VEGF [26]. The different effects on endothelial cell proliferation of early and late activated M ϕ were also investigated. And the results showed that the proliferation index in early-activated M ϕ -treated group was almost twice as high as that in late-activated M ϕ -treated group. However, early apoptosis and late apoptosis/necrosis of ECV304 in late-activated M ϕ -treated group was more significant than that of early-activated M ϕ -treated group. All the results showed that the influences were more markedly in contacting co-culture system than that in non-contacting co-culture system. Apoptosis occurs normally in development and homeostasis is balanced with that stimulation after cytotoxic insult, metabolic imbalance, or infectious attack. Human cells commit to apoptosis *via* one of two generalized activation pathways: First, death receptor-independent deregulation of mitochondrial function, during which cytosolic cytochrome *c* binds with apoptotic protease activating factor-1 (APAF-1) and cleaved procaspase-9 to form the apoptosome. Second, activation of the death receptor pathway through ligation of CD95 (FAS/APO-1), tumor necrosis factor (TNF)- α receptor 1, or other death receptors at the eukaryotic cell surface may lead to the activation of caspase-8, cleavage of procaspase-3 (or effector caspases-6 or -7), and terminal extrinsic apoptosis events [27]. In further study, the specific mechanism of endothelial cell apoptosis which is affected by the early or late activated M ϕ would be elucidated.

There are several viable methods to measure motility *in vitro*. Boyden chamber assay could provide a convenient method to assess cell migration and quantitative data [28]. So the endothelial cell motility assay was carried out using a modified Boyden-chamber assay system. The number of ECV304 cells migrating through the membrane was determined using the colorimetric crystal violet (CV) assay. The crystal violet colorimetric assay could directly measures the cell numbers [29]. The results of the present study demonstrated that

early-activated Mφ could promote the migration of ECV304 more significant than that of late-activated Mφ. And in further study, the different levels of relevant chemokines would be tested.

As we can see that all the influences of lipopolysaccharide mediating early- and late-activated THP-1 macrophages on ECV304 endothelial cell dysfunction were more markedly in contacting co-culture system than that in non-contacting co-culture system, especially for the apoptosis. Consistent with previous research, direct cellular contact could induce apoptosis [30]. Two types of effector/target contacts were seen: either effector cell protrusions were pushed deep into pouches and lacunae of the target cell surface, or a wide area of intimate cell-to-cell contact was formed [31]. The appearance of anionic phosphatidylserine on the cell surface is the most remarkable feature in apoptosis because of the abundance of this phospholipid in membranes, its negative charge and the ability to change interactions with other lipids leading to disruption of lipid rafts [32]. This may be one of the mechanism of cellular contact-induce apoptosis in present study and the corresponding research will be carried out in the further.

Conclusions

The present study suggested that the early-activated Mφ could promote the secretion of VEGF and proliferation and migration of ECV304, whereas the late- activated Mφ could promote the apoptosis and necrosis of ECV304 in contacting system.

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