Dynamic monitoring of changes in endothelial cell-substrate adhesiveness during leukocyte adhesion by microelectrical impedance assay

Yakun Ge, Tongle Deng, and Xiaoxiang Zheng*

Key Laboratory for Biomedical Engineering of Ministry of China, Department of Biomedical Engineering, Zhejiang University, Hangzhou 310027, China
*Correspondence address. Tel/Fax: +86-571-87953860; E-mail: zxx@mail.hz.zj.cn

Adhesion of leukocytes to endothelial cells in inflammation processes leads to changes of endothelial cell-substrate adhesiveness, and understanding of such changes will provide us with important information of inflammation processes. In this study, we used a non-invasive biosensor system referred to as real-time cell electronic sensor (RT-CES) system to monitor the changes in endothelial cell-substrate adhesiveness induced by human monoblastic cell line U937 cell adhesion in a dynamic and quantitative manner. This assay, which is based on cell-substrate impedance readout, is able to monitor transient changes in cell-substrate adhesiveness as a result of U937 cell adhesion. The U937 cell adhesion to endothelial cells was induced by lipopolysaccharide (LPS) in a dose-dependent manner. Although the number of adherent U937 cells to the endothelial cells was verified by a standard assay, the adhesiveness of endothelial cells after addition of U937 cells was monitored by the RT-CES system. Furthermore, focal adhesion kinase protein decrease and F-actin rearrangement in endothelial cells were observed after addition of U937 cells. Our results indicated that the adhesion of U937 cells to LPS-treated endothelial cells reduced the cell adhesiveness to the substrate, and such reduction might facilitate infiltration of leukocytes.

Keywords dynamic monitor; label-free; cell-substrate adhesiveness; RT-CES

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Introduction

Adhesion of circulating leukocytes to endothelium and their migration into the perivascular tissues are fundamental components of normal physiology such as inflammation as well as pathophysiological events such as the initial stage of atherosclerosis [1]. The dynamic nature of leukocyte adhesion and migration involves leukocyte binding and cell-substrate adhesiveness changes in endothelial cells due to rearrangement of the focal adhesion complex and the actin cytoskeleton [2,3]. It is a highly regulated process that requires the participation of specialized cell surface receptors, signaling proteins, and cytoskeleton [4–6]. Anti-platelet/endothelial cell adhesion molecule-1 antibody inhibits the transmigration of leukocytes in vitro without interfering with the leukocyte’s potential to adhere to endothelial cells [7]. CD47, present on both neutrophils and endothelial cells, is supposed to be essential for invasion [8]. Activation of intercellular adhesion molecule-1 by binding of T cells has been reported to induce tyrosine phosphorylation of the actin-binding protein, indicating alterations in the cytoskeleton [9]. These findings suggest the possibility that binding itself induces changes in endothelial cells leading to relaxation of focal contacts.

To understand the biology of leukocyte adhesion, it is necessary to have a stable and reproducible assay system. Traditional cell-adhesion methods are based on enzymatic approaches, in which fluorescent probes [10,11] and non-fluorescent probes [12] are widely used. These assays involve extensive handling of the cells, cell fixation and labeling, and manual counting. In addition, all of the above-mentioned methods are end-point assays and do not provide crucial information regarding the dynamics of leukocyte adhesion.

To overcome some of the limitation in current assay systems, we develop a label-free and dynamical method to monitor leukocyte adhesion. The method is based on non-invasive measurement of endothelial cell-substrate adhesiveness. A real-time cell electronic sensing
(RT-CES; Roche Applied Sciences, Basel, Switzerland) system was applied to detect transient changes of endothelial cell-substrate adhesiveness. This system measures changes in cell-substrate impedance as a result of the disruption of the ionic environment due to cell spreading, adhesiveness, mobility, morphology, and barrier function [13–15]. It has been successfully applied to monitor changes mentioned above in various cell types under different experiment conditions [12–14,16]. The cellular status is continuously monitored using the RT-CES system.

In this study, we detected changes of endothelial cell-substrate adhesiveness dynamically by the RT-CES system, measured the amount of focal adhesion kinase (FAK) on endothelial cells by fluorescence photometric detection, and observed changes of F-actin stress fibers of endothelial cells under fluorescent microscopy during leukocyte-endothelium adhesion in vitro.

Materials and Methods

Materials

Culture media and reagents were purchased from Gibco (Gaithersburg, USA), and tissue culture plates were from Corning (NY, USA). The e-plates for impedance measurements were purchased from Roche Applied Sciences. Lipopolysaccharide (LPS) was purchased from Sigma Chemical (St Louis, USA). BODIPY FL phallacidin and 2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM) were obtained from Molecular Probes (Carlsbad, USA). The rabbit anti-p125FAK monoclonal antibody was purchased from Epitomics (USA), and FITC-conjugated goat IgG was purchased from Santa Cruz (Santa Cruz, USA). All other chemicals were of the highest grade of purity and were commercially available.

The RT-CES system is composed by three parts: an electronic sensor analyzer, a device station, and 16-well e-plates [7]. The e-plate containing tissue culture well is consistent with dimensions of standard flat-bottom 96-well culture plate. The well bottom has incorporated circle-on-line sensor electrode arrays (circle diameter 90 μm, line with 30 μm, and line-to-line spacing 110 μm). The gold microelectrodes cover ~80% area of the bottom. Only 16-well e-plates were used in our study. The device station, which is connected with e-plates, is placed in the incubator and connected to the electronic sensor analyzer through electrical cables.

Cell index (CI) is a parameter used to represent cell status based on the electrical impedance measurements, which is calculated of frequency-dependent impedance according to the formula:

$$CI = \max_{i=1…N} \left( \frac{R_{cell}(f_i)}{R_{b}(f_i)} - 1 \right)$$

where $R_b(f_i)$ and $R_{cell}(f_i)$ are the frequency-dependent electrode resistances without or with cells present, respectively, at different frequencies ($n$ is the number of frequency points at which the impedance is measured, i.e., $n = 3$ for 10, 25, and 50 kHz) [17]. Each frequency is applied for ~100 ms to each well. CI is a relative value to indicate how many or how cell attached to the electrodes. Under the control of the RT-CES software, experiment data are measured automatically by the sensor analyzer.

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins as described previously [18]. The endothelial cells were cultured in RPMI 1640 medium containing 20% fetal calf serum (FBS) and vascular endothelial growth factor (10 ng/ml). The human leukemia monocyte lymphoma cell line U937 was purchased from American Type Culture Collection (ATCC, Manassas, USA). U937 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. The cells were maintained at 37°C in humidified air with 5% CO₂.

Observation of F-actin in HUVECs

HUVECs were plated on coverslips and grown until confluence. These cells were treated with LPS for 6 h and subsequently layered with U937 cells at different cell densities for indicated times. Then HUVECs were rinsed with phosphate buffered solution (PBS) and fixed in 3.7% paraformaldehyde for 10 min at room temperature. After fixation, the cells were treated with 0.2% EDTA for ~1 min to remove the bounded U937 cells. Subsequently, cells were permeabilized in 0.1% Triton X-100 for 5 min at room temperature and then blocked with 1% BSA in PBS for 30 min. The cells then were washed in PBS and incubated with 1 U BODIPY FL phallacidin. Finally, the cells were washed and observed by Confocal laser scanning microscopy (Zeiss LSM510, Jena, Germany).

CI measurement during leukocyte-endothelial cell adhesion in vitro

For real-time calculation of CI, 50 μl of cell culture medium was added into each sensor well in order to measure the background readings, followed by the addition of 100 μl of HUVEC suspension at the concentration of 5 × 10⁵ cells/ml. The e-plates containing
HUVECs were allowed to incubate at room temperature for 10 min before being placed on the device station in the incubator for continuous recording of CI. The HUVECs were allowed to attach and spread overnight to reach a stable baseline, and the cells were treated with LPS. After 6 h, U937 cells were added into individual wells at different cell densities for the indicated time. All these measurements were collected by analyzer with an interval of 5 min.

**Fluorescence photometric detection on FAK level on HUVECs**

HUVECs were plated on black sided, clear bottomed, 96-well plates (Greiner Bio-One, Stonehouse, UK) for FlexStation assays and grown until confluence. These cells were treated with LPS for 6 h and subsequently added with U937 cells for the indicated time. After washing the mixed cultures of HUVECs and U937 cells with PBS, the cells were fixed in 3.7% paraformaldehyde for 10 min at room temperature, and the cells were treated with 0.2% EDTA for ~1 min to remove the bound U937 cells. Subsequently, cells were permeabilized in 0.1% Triton X-100 for 5 min at room temperature and then blocked with 1% BSA in PBS for 30 min. Finally, the cells then were washed in PBS and incubated with anti-p125FAK mAb (1:150 in PBS) and FITC-conjugated goat IgG (1:1000 in PBS). These cells were washed in PBS before fluorescence assay. Fluorescence was measured using FlexStation II (Molecular Devices, Sunnyvale, USA). All experiments were performed at 20°C. A Xe lamp provided excitation light and monochromators was used to select excitation (485 nm) and emission (520 nm) wavelengths.

**Quantification of adherent leukocyte**

Confluent HUVECs were treated with LPS for 6 h prior to the assay. U937 cells were loaded with 1 μM of BCECF-AM in dark for 1 h at room temperature, washed three times with culture medium, and suspended in culture medium. After incubation with labeled U937 cells for indicated time at 37°C, HUVECs were gently washed twice with PBS; and the number of adherent U937 cells was counted by fluorescence microscopy.

**Statistical analysis**

Results are expressed as the mean ± SD. Statistical comparisons between groups were carried out by an unpaired Student’s t-test. P-value of <0.05 was considered indicative of a statistically significant difference.

**Results**

**Kinetics of the changes in CI, FAK amount, and adherence of U937 cells in LPS-treated HUVECs**

To mimic the activated status of blood vessels in inflammation, HUVECs were pretreated with LPS at a concentration of 4 ng/ml for 6 h. **Figure 1(A)** shows the changes of normalized CI of HUVECs during U937 binding. There was almost no impact of LPS application alone on the CI of HUVECs. After application of U937 cells, the CI of LPS-activated HUVECs decreased gradually, whereas that for the unstimulated HUVECs was almost unchanged. After 10 min of application of U937, there was already a statistically significant difference between the two groups. The number of adherent U937 and FAK amount was measured from 5 to 40 min after U937 addition. As shown in **Fig. 1(B)**, the decrease in p125FAK was detected from 5 min after the application of U937 and reached a low level 10–40 min later. The number of bound U937 increased in a time-dependent manner and maintained at a high level 10–40 min later [**Fig. 1(C)**].

![Fig. 1](image_url)
Effect of LPS treatment of HUVECs on the changes in CI and FAK in HUVECs during U937 binding

Exposure of HUVECs to LPS results in leukocytes tethering, rolling, and firm adhesion on endothelial cells. We investigated the changes in endothelial cell-substrate adhesiveness and the protein level of p125FAK after adding U937 cells. HUVECs were stimulated with LPS at different concentrations (1–64 ng/ml) for 6 h and were co-cultured with U937 for 40 min. As shown in Fig. 2(A), decrease in CI value was observed in HUVECs in a manner dependent on the concentrations of LPS, and there was a clear dose-dependent effect of LPS on the decrease in p125FAK amount [Fig. 2(B)].

Changes in CI, adherence of U937, and FAK in LPS-treated HUVECs induced by U937 binding

HUVECs were pretreated with LPS at the concentration of 4 ng/ml for 6 h. Subsequently, U937 cells at varying densities (0.375–24 × 10⁴ cells/ml) were added into HUVECs, the changes in CI were recorded by the RT-CES system. After co-culture of HUVECs for 40 min, the HUVECs were treated for U937 binding and p125FAK amount detections. As shown in Fig. 3, CI of HUVECs decreased in a density-dependent manner. Increased number of binding U937 presented a density-dependent profile, and so did the decrease in p125FAK amount.

As shown in Figs. 1(A), 2(A), and 3(A), the addition of U937 cells to LPS-activated HUVECs promptly elicited a decrease in CI. To understand CI in relation to leukocytes binding to endothelial cells, we measured the number of binding U937 cells. The correlation and regression analyses showed a good negative linear correlation between CI and the natural logarithm of the number of adherent U937 cells [Fig. 3(D), \( R = -0.9911 \)].

Changes in F-actin distribution in LPS-treated HUVECs induced by U937 binding

To investigate the effect of U937 binding on the cytoskeletal structure of endothelial cells, HUVECs were treated with or without LPS for 6 h and were overlayered with U937 cells for 40 min. Treatment of HUVECs with 4 ng/ml LPS for 6 h resulted in the disappearance of the dense peripheral bands of F-actin and the formation of stress fibers in the cytoplasm [Fig. 4(A,B)]. As the fluorescence photomicrographs showed in Fig. 4(A,C), U937 cells adherence to untreated HUVECs had little effect on the F-actin distribution in HUVECs. U937 adherence to LPS-treated HUVECs induced the formation of F-actin clusters [Fig. 4(D)].

Discussion

In the present study, we used RT-CES system to assay endothelial cell-substrate adhesiveness during leukocyte binding. We found that application of U937 cells to LPS-treated HUVECs immediately decreased the endothelial cell-substrate impedance and FAK amount. RT-CES is a label-free in vitro technique for cell behavior study. The basic principles have been known for many years [19,20]. And now there are several commercially available systems such as the electrical cell-substrate impedance sensing system and RT-CES. CI is the parameter to represent cell status based on the measured electrical impedance. The calculation of
frequency-dependent impedance and corresponding CI value has been described in detail in the Materials and methods section. Since the fractional contribution of cell-substrate contacts to the electrical impedance is strictly dependent on the frequency of the sensing voltage, within the intermediate frequency window (10 Hz < f < 100 kHz) the impedance varies for different frequencies [21] and shows different sensitivity to the changes in impedance. All the impedance measurements reported here were made at frequencies of 10, 25, and 50 kHz. At these frequencies, the difference between the measured impedance of the electrode with and without cells is close to maximum for various cell types. It allows for very small variations in impedance due to changes in the cell layer to be detected.

According to the formula described in the Materials and methods section, several features of the CI can be derived. Under the same physiological conditions, the more cells attach to the electrodes, the higher impedance value leading to a larger CI value. When the same number of cells attach to the electrodes, different cell status will lead to different values of CI. An increase in cell adhesion or cell spread leading to a larger cell-substrate contact area lead to an increase in CI. On the other hand, cell death, detachment, or rounding up will lead to a decrease in CI. Therefore, CI is a measure of both the total contract area of cells on the electrodes and the strength of cell adhesion to the electrodes [12,13]. The changes in the CI value reflected the changes in cell adhesion.

The decrease in CI may be related with the change of the adhesiveness of endothelial cells to the substrate. The amount of p125FAK in HUVECs decreased after adhesion of U937 cells in the manner dependent on LPS concentrations or U937 densities [Figs. 1(B), 2(B), 3(B)]. FAK, which is a kind of tyrosine kinase localized in focal contacts that form a link between extracellular matrix and actin cytoskeleton, plays an important role in the endothelial cell attachment to the extracellular matrices [2,22]. The inhibition of p125FAK function

**Fig. 3** Changes in CI, FAK level and number of adherent U937 on HUVECs induced by co-culture with U937. Confluent HUVECs were treated with 4 ng/ml LPS for 6 hr and were subsequently overlaid with 0.375-24×10⁵ cells/ml U937 for 40 min. (A)Summary of CI changes in LPS-activated HUVECs (n = 4). The arrow indicates the application of U937. (B) Decrease of FAK decrease induced by U937 binding (n = 6). (C) Number of adherent U937 on the surface of 1mm² HUVECs monolayer (n = 5). (D) The relationship between number of adhesion U937 on the surface of 1mm² HUVEC monolayer and CI at 40 min after U937 addition (n = 6). *P < 0.05 vs. control (HUVECs overlaid with 0 cells/ml U937); **P < 0.01 vs. control.
blocked the formation of focal contacts [20], and the loss of p125FAK was a prerequisite for cell detachment [3]. In addition, it has been reported that the reduction of focal contacts agreed well with the decrease in CI, the parameter of cell-substrate impedance in the RT-CES system [15]. It has been well known that the formation of actin stress fibers parallels the formation of focal adhesion [23,24] and is closely related to morphology formation of endothelial cells [25]. As shown in Fig. 4, F-actin filaments are densely composed in the peripheral region of HUVECs before U937 application. The reduction of F-actin filaments may cause the changes in endothelial cell shape. Therefore, the decrease in FAK [Figs. 1(B), 2(B), and 3(B)] and reorganization of F-actin stress fiber (Fig. 4) indicate the decrease in endothelial cell-substrate adhesiveness, which results in the decrease in CI [Figs. 1(A), 2(A), and 3(A)]. The cellular adhesiveness is probably decreased during the morphological changes and rearrangement of the focal contacts of endothelial cells. Because endothelial cells need to change their shape dynamically during U937 transendothelial migration, the endothelial cells may need to reduce their adhesiveness to the bottom of e-plates in the process of U937 adhesion.

In conclusion, using RT-CES system and immunofluorescence, we demonstrated that U937 adhesion reduced the cell-substrate adhesiveness of endothelial cells, which is caused by p125FAK amount loss and actin filaments rearrangement. The adhesion of leukocytes plays a key role in many physiological and pathologic processes by influence signal transduction pathway in endothelial cells and by inducing rearrangement of the focal adhesion complex and actin stress fibers, resulting in the facilitated migration of leukocytes.
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References

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