Label-free and Dynamic Monitoring of Endothelial Barrier Function on Microelectronic Cell Sensor Arrays

Han-Bing Li[1,2], Tong-Le Deng[1], Qing-Nan Zhang[1], Xiao-Xiang Zheng[1]*

([1]*Department of Biomedical Engineering, Zhejiang University, Hangzhou 310027, China;*
[2]*College of Pharmaceutical Sciences, Zhejiang University of Technology, Hangzhou 310032, China*)

Abstract The endothelium plays a vital role in the maintenance of vascular homeostasis and barrier dysfunction is a characteristic of many inflammatory conditions. In our study, an online and continuous technique based on real-time cell electronic sensing (RT-CES) was introduced into study of endothelial barrier function. Electrical impedance in human umbilical vein endothelial cells (HUVECs) was monitored in real-time on microelectronic cell sensor arrays using RT-CES from seeding to confluence. α-thrombin induced electrical impedance alteration in a dose-dependent manner which was paralleled by transendothelial FITC-dextran permeability across endothelial monolayer. Correspondingly, F-actin cytoskeleton redistribution of HUVECs accompanied the change of electrical impedance. The results suggest the RT-CES system could be a powerful tool to study endothelial barrier function model *in vitro*.

Key words endothelial cell; barrier function; RT-CES; permeability; F-actin

The vascular endothelium plays a central role in the maintenance of vascular homeostasis, which is required to control the flow of plasma components and circulating cells between the blood and subendothelial compartments. A decrease in the barrier properties of vascular endothelium leads to tissue edema. Increased endothelial permeability to plasma proteins is the characteristic of many inflammatory conditions. Inflammatory mediators such as histamine, bradykinin, and α-thrombin cause a rapid transient increase in permeability *in vivo*, which results from a rapid formation of endothelial gaps especially in the postcapillary venules[1].

Endothelial cells achieve this semi-permeability barrier by interacting with each other in a dynamic fashion through specific receptors called cadherins or occludins, which are homotypic receptors that interact with each other at specialized junctions in the membrane called the adherens junctions or tight junctions, respectively[2,3]. Inflammatory mediators induce breakdown of the endothelial barrier by interacting with specific cell surface receptors on endothelial cells and transmitting signals to the endothelial cytoskeleton. Cytoskeletal changes can influence the overall morphology of the cells and can induce breakdown of cell-cell junctions, which are critical for maintaining the endothelial barrier function[4].

There are a number of researchers who are interested in investigating the molecular mechanisms of endothelial barrier function both from a basic research point of view as well as for drug discovery. A number of label-based assays are currently available for studying endothelial barrier function. These label-based assays utilize trans-wells on which a confluent layer of endothelial cells are seeded and a tracer dye or label is added into the upper compartments[5]. Alternatively, transendothelial resistance (TER) measurement prior and subsequent to a certain treatment is another means of determining the permeability of the endothelial monolayer[6].

The assays described above provide limited information in its utility in terms of the time it takes to set up and process the experiment and due to labeling of the

Received: April 24, 2006 Accepted: June 20, 2006

This work was supported by the Key Laboratory of Biomedical Engineering, Ministry of Education and the Key Laboratory of Chinese Medicine Screening, Exploitation and Medicinal Effectiveness Appraise for Cardio-cerebral Vascular and Nervous System of Zhejiang Province

*Corresponding author. Tel: 86-571-87953860, Fax: 86-571-87951676, E-mail: zxx@mail.hz.zj.cn

cells which can produce high background and low reproducibility. Most importantly, the assays mentioned above are endpoint assays, while the process of endothelial barrier function disruption is a dynamic and transient process. Therefore, what is needed is a method that not only measures disruption of the barrier function, but can be performed in real-time in order to appreciate and understand the full dynamics of the process.

In this application we describe a novel cell-based assay for monitoring barrier function using real-time cell electronic sensing (RT-CES) system. The major advantage of this method over traditional assays is its label-free and real-time conducting so kinetic information regarding the assay can be obtained. Furthermore, the assay readout relies on measurement of morphological dynamics which is an inherent cellular response to agents that modulate the barrier function and therefore precludes the need for reporters or labeling.

1 Materials and Methods

1.1 Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated by the method of Jaffe *et al.*[7] and characterized as described previously. Cells were cultured on flask in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 20% newborn calf serum, 30 μg/ml endothelial cell growth factor (Sigma), 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were kept at 37 ℃ under 5% CO_2 and 95% air. Confluent cells were used between the second and the fourth passages.

1.2 RT-CES experimental setup

The detailed experimental procedures were conducted as described previously with some modifications[8]. Briefly, 100 μl of growth media was gently dispensed into a 16-well strip for background readings by the RT-CES system prior to addition of 100 μl of cells at 2×10^5/ml. Devices containing cells were kept at room temperature in a tissue culture hood for 10 min prior to insertion into the RT-CES device in the incubator for continuous recording of impedance as reflected by cell index (CI, see detailed definition below). Cells were allowed to attach, spread and grow to confluence before stimulations. The results were expressed by normalized CI, which is derived from the ratio of CI at the time point and at a reference point.

1.3 Impedance and CI measurements by RT-CES system

The RT-CES system (ACEA Biosciences, San Diego, CA) is comprised of three components: an electronic sensor analyzer, a device station, and a 16-well strip. The circle-on-line electrode arrays, specially designed and optimized for its improved signal strength when cells are attached, are fabricated on glass slides with lithographical micro fabrication methods. The electrodes-containing chip is assembled on plastic trays so that the electrode sensor array forms the well bottom (Fig.1). The device station holds the 16-well strip and is capable of electronically switching any one of the wells to the sensor analyzer for impedance measurement.

To quantify cell status based on the measured cell-electrode impedance, a parameter termed CI is derived, according to formula:

$$CI=\max_{i=1,2,\cdots\cdots N}\left(\frac{R_{cell}(f_i)}{R_0(f_i)}-1\right)$$

where $R_0(f)$ and $R_{cell}(f)$ are the frequency-dependent electrode resistance without cells or with cells present in the wells, respectively. N is the number of the frequency points at which the impedance is measured.

1.4 Permeability test

Confluent cells were trypsinised and seeded in a high density onto Millicell polycarbonate membrane as-

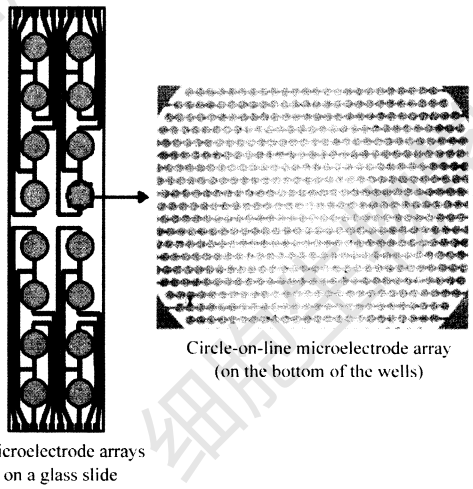

Fig. 1 A schematic illustration of a 16-well device with 16 circle-on-line electrode array units

semblies (0.33 cm^2, 3 μm pore size; Millipore) suspended in 24-well culture plates. Permeability tests were conducted as described by Langeler *et al.*[9] with slight modifications. The permeability of the endothelial monolayer is assessed by measuring the rate of redistribution, from the inner chamber of the filter inserts to the outer chamber in the petri dishes, of fluorescein isothiocynate-labeled dextran (40 kDa, Sigma). 5 mg/ml of FITC-dextran in DMEM was added to the upper compartment of the filter inserts in the presence or absence of thrombin. Samples were taken from the lower compartment at the indicated times for permeability assessments.

1.5 Phalloidin staining and confocal microscopic imaging

F-actin was stained according to the method of Yu *et al.*[10] with slight modifications. HUVECs were grown to confluence on glass coverslips and treated for the indicated time period. The cells fixed with 3.7% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 5 min and incubated for 30 min at 37 ℃ with bodipy-labeled phalloidin (Molecular Probe, USA) and propidium iodide (PI) and viewed with the Zeiss LSM 510 confocal microscope.

1.6 Statistical analysis

Data were represented as $\bar{x}\pm s$. The statistical comparison between groups was carried out using Student's *t* test. *P* values<0.05 were considered significant.

2 Results

2.1 Real-time monitoring of electrical impedance in HUVECs from seeding to confluence

As shown in Fig.2, endothelial impedance displayed as normalized CI in HUVECs monolayer was monitored using RT-CES from seeding to confluence and showed only tiny variation between different wells.

2.2 Real-time monitoring of barrier dysfunction induced by thrombin in HUVECs

The attachment, spreading, growth and proliferation of the cells were monitored using the RT-CES system to make sure the cells have formed a confluent layer which shows up as a cell trace which is level and does not increase or decrease over time, then α-thrombin was added. As shown in Fig.3, α-thrombin induced a rapid decrease dose-dependently in endothelial electrical impedance displayed as CI, which was maximal after 20 min compared with basal level, then recovered to the basal level gradually.

We also measured transendothelial FITC-dextran permeability, a direct measure of endothelial barrier function[11] to assess endothelial barrier function. The decrease in electrical impedance was paralleled by the increase in the passage of FITC-dextran across endothelial monolayer at the time point of 20 min after addition of α-thrombin which also has concentration-dependent profile (Fig.4).

2.3 Electrical impedance alteration was accompanied by cytoskeletal remodeling

The behavior of F-actin microfilaments of endothelial cells following stimulation with inflammatory agonists plays a vital role in the formation of gaps between adjacent endothelial cells. Confluent HUVECs before treatment typically displayed few stress fibers (Fig.5A) and exhibited a thin band of F-actin along cell margin, in apposition with the adjacent cells. The F-actin filaments underwent a rapid redistribution after stimulation with α-thrombin. Fig.5B and Fig.5C show distribution pattern of F-actin filaments in HUVEC after application of 0.625 u/ml α-thrombin for 5 min and 20 min respectively. By 5 min of application of α-thrombin, an increase in amount of F-actin filament was observed. Meanwhile, stress fibers were also observed in the cell periphery around the nucleus and occasionally across the nuclear region. By 20 min of application of α-thrombin, F-actin filaments were prominent around the cells. These results coincide with previous reports that change in the content and redistribution of F-actin filaments lead to cell contraction, formation of gaps and barrier function aberration[12,13].

3 Discussion

There are several methods for assessing and quantifying endothelial barrier function. The traditional method is to assess quantitative penetration of a tracer dye , either fluorescent or chemiluminescent, from the inner chamber to outer chamber through transwell

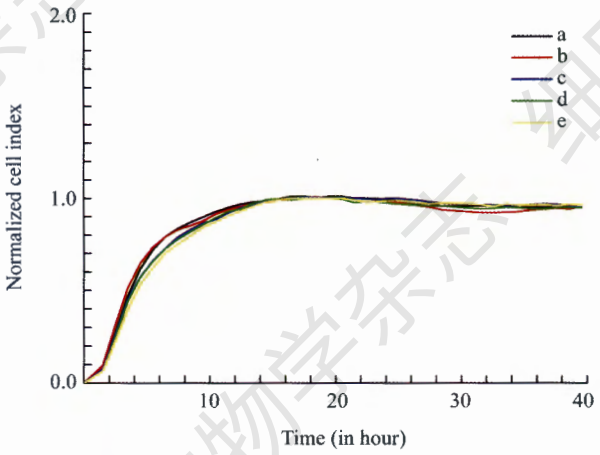

Fig. 2 Real-time monitoring of electrical impedance in HUVECs on microelectronic sensors

a–e represent different wells.

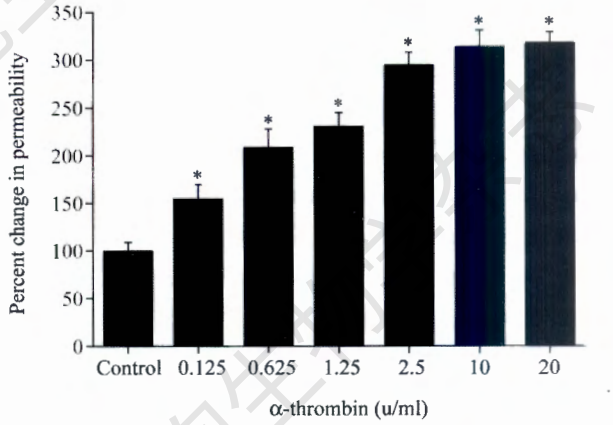

Fig. 4 Transendothelial permeability alteration induced by α-thrombin

Values are shown as $\bar{x}\pm s$ (n=6), $^*P$<0.01, versus control group.

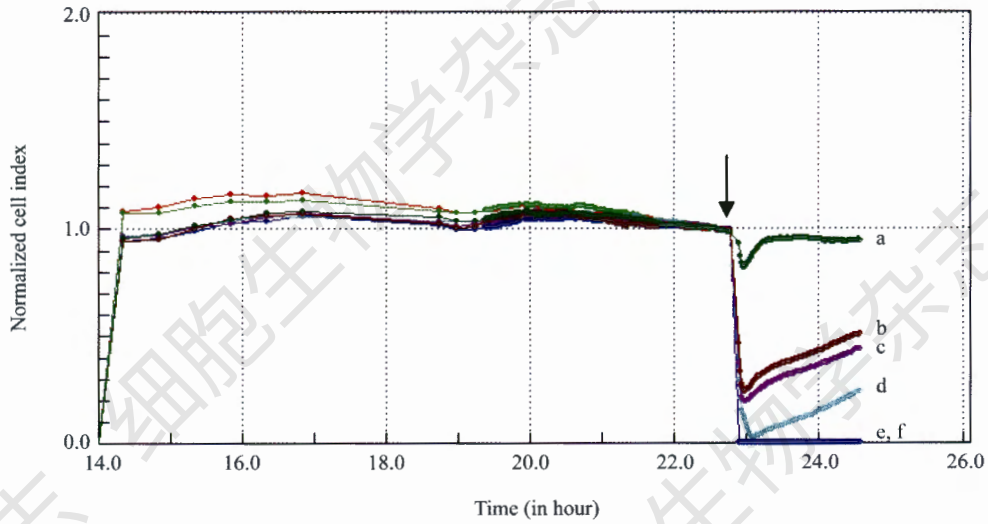

Fig. 3 Real-time monitoring barrier function in HUVECs on microelectronic sensors stimulated with different concentrations of α-thrombin

a–f represent 0.125, 0.625, 1.25, 2.5, 10 and 20 u/ml α-thrombin respectively. The arrow indicates the addition of α-thrombin.

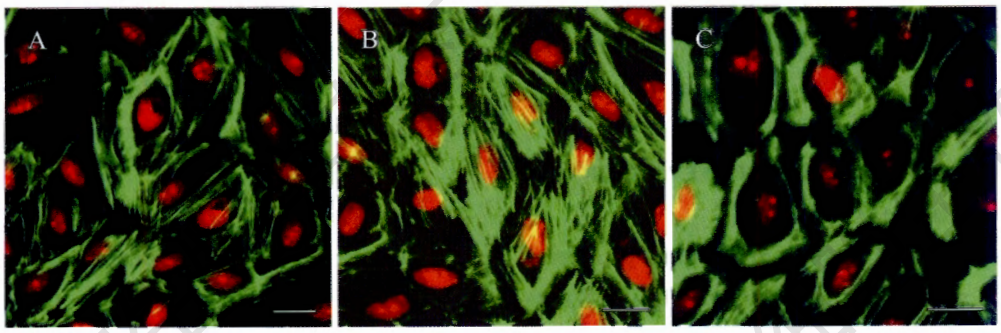

Fig. 5 F-actin staining

A: F-actin filaments pattern in untreated HUVECs; B, C: F-actin filaments pattern in HUVECs 5 min and 20 min after application of 0.625 u/ml α-thrombin respectively. Scale bar represents 20 μm.

permeable supports. Meanwhile, TER measurement before and after application of certain treatment was also used to demonstrate permeability alteration and barrier function of endothelial monolayer. Alternatively, the application of electrical cell-substrate impedance sensing (ECIS) for measurement of cellular processes was first reported by Giaever *et al.*[14] and used to monitor endothelial cell shape change for assessment of its barrier function[15]. However, it has a big limitation. Only a small percentage of the cells (<0.063% well bottom surface area) in a given well which attach to the detection electrodes, are monitored and measured in the ECIS system. This leads to relatively large well-to-well variations in the impedance signals because of the uneven distribution of cells that initially settle onto the detection electrodes[16].

In the present study, we introduced a new method to monitor endothelial barrier function, which is not only sensitive, label-free and quantitative, but also overcome the shortcoming of large variation. In the ACEA RT-CES system, microelectrode sensor array was fabricated on glass slides with lithographical microfabrication methods, and the electrode-containing slides are assembled to plastic trays to form electrode-containing wells. In contrast to other impedance sensors[17,18], the novel design of the electrode, termed circle-on-line electrode array, covers approximately 80% of the surface area of the well, which lead to consistent and reproducible results[8].

The basic principle of the RT-CES system is similar to the electrical cell impedance detection system previously described, such as those of ECIS[19]. Electronic impedance of an electrode is determined basically by the ion environment both in the bulk solution and at the interface between the electrode and the solution. Totally, the resistance of the solution (so small that can be ignored) and the impedance at the interface constitute the electrode impedance. The attached and spread cells often act as insulating particles because of their plasma membrane to interfere with the free space immediately above the electrode for current flow[20]. Thus, the more cells attaching on the electrode, the more increase in the electrode impedance. Furthermore, the impedance change is also influenced by the extent of cell attachment, spread and proliferation. As shown in Fig. 2, electrical impedance increases as cell proliferates till confluence. When the cells on the electrode reach confluence, the electrode impedance primarily depends on cell attach and shape. Any stimulation causing cell contraction and formation of gaps between cells can lead to the electrical impedance change.

In conclusion, the RT-CES system can be applied to monitor barrier function of endothelial cells on microelectronic cell sensor arrays and have advantages over methods reported previously. Real-time monitoring and the preclusion of labeling reagents provide succinct information concerning endothelial barrier alteration induced by stimulation and kinetics involved in the process. Furthermore, the readout is noninvasive, cell attachment and proliferation can be assessed in the same wells before application of stimulation. These enable RT-CES system a powerful tool to study endothelial barrier function model *in vitro*.

Acknowledgements The authors thank Xiao Xu, Ph D. and ACEA Biosciences Incorporation (San Diego, California, USA) for the generous support to this study.

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利用微电子细胞传感器阵列动态监测内皮屏障功能

李汉兵[1,2] 邓同乐[1] 张清楠[1] 郑筱祥[1]*

([1] 浙江大学生物医学工程系, 杭州 310027; [2] 浙江工业大学药学院, 杭州 310032)

摘要 内皮在保持血管的动态平衡中起着至关重要的作用, 而内皮屏障的功能损伤是很多炎症反应的重要特征。利用实时细胞电子分析系统体外研究脐静脉内皮的屏障功能, 从细胞的贴附、生长到融合状态, 实时监测了微电子细胞传感器阵列上细胞阻抗的动态变化。α-凝血酶以剂量依赖的方式显著诱导单层内皮细胞阻抗快速下降, 而后缓慢回升。相应地, α-凝血酶显著引起细胞单层对荧光标记葡聚糖通透性的改变和F-肌动蛋白细胞骨架的重分布。结果表明, 实时细胞阻抗系统可能成为一种体外研究血管内皮细胞形态和屏障功能的有力的手段。

关键词 内皮细胞; 屏障功能; 实时细胞电子分析系统; 通透性; F-肌动蛋白

收稿日期: 2006-04-24 接受日期: 2006-06-20

教育部生物医学工程重点实验室和浙江省心脑血管、神经系统药物评价和中药开发重点实验室资助

* 通讯作者。Tel: 0571-87953860, Fax: 0571-87951676, E-mail: zxx@mail.hz.zj.cn