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Use of electric cell–substrate impedance sensing to assess in vitro cytotoxicity

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ABSTRACT

In vitro assessment of cytotoxicity based on electrochemical impedance spectroscopy (EIS) needs more quantitative methods to analyze the alteration of cell morphology and motility, and hence the potential risk to human health. Here, we applied electric cell–substrate impedance sensing (ECIS) to evaluate dose-dependent responses of human umbilical vein endothelial cells exposed to cytochalasin B. To detect subtle changes in cell morphology, the frequency-dependent impedance data of the cell monolayer were measured and analyzed with a theoretical cell–electrode model. To detect the alternation of cell micromotion in response to cytochalasin B challenge, time-series impedance fluctuations of cell-covered electrodes were monitored and the values of power spectrum, variance, and variance of the increments were calculated to verify the difference. While a dose-dependent relationship was generally observed from the overall resistance of the cell monolayer, the analysis of frequency-dependent impedance and impedance fluctuations distinguished cytochalasin B levels as low as 0.1 μM. Our results show that cytochalasin B causes a decrease of junctional resistance between cells, an increase of membrane capacitance, and the reduction in micromotion.

1. Introduction

Among various cell-based assays, electrochemical impedance spectroscopy (EIS) using microelectrode arrays has emerged as a promising label-free method for detecting cellular responses to toxins of chemical or biological origin (Ceriotti et al., 2007; Keese et al., 1998; Slaughter et al., 2004; Xing et al., 2005). Electrochemical impedance measurements using microelectrodes have been first used to study the characteristics of anchorage dependent cultured cell lines by Giaever and Keese (1984). The electric cell–substrate impedance sensing (ECIS) is a device developed by them that monitors the impedance of small gold electrodes used as substrata for cells in culture. The system has been extensively studied and can be used to detect subtle changes in the cell–substrata interactions including cell motion. Using this device, Giaever and Keese and other ECIS users have been able to measure cell attachment and spreading (Mitra et al., 1991), cell motility (Giaever and Keese, 1991; Lo et al., 1993), barrier function of cell layers (Tiruppathi et al., 1992), and in vitro toxicology (Keese et al., 1998; Ko et al., 1998; Xiao et al., 2002).

For ECIS and other EIS systems, the most commonly used assay for cytotoxicity test is to take the time course of overall impedance of a cell-covered electrode and then derive the half-inhibition concentration from the response function. It can be employed to follow cell attachment and spreading in response to different toxin concentrations and exposure time (Xiao et al., 2002). In this method, the toxic compound is added to the cell suspension right before cells are inoculated to electrode wells. Another way is to seed cells over the electrode wells until a confluent monolayer is achieved. Toxic compound is then added to the cell monolayer and impedance changes of the cell-covered electrode are followed (Ceriotti et al., 2007; Ehret et al., 2001; Xing et al., 2005; Yeon and Park, 2005). It has been shown that the half-inhibition concentrations determined by EIS assays agreed well with the values determined with standard biochemical methods, such as neutral red uptake (NRU), (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, lactate dehydrogenase (LDH) measurement, colony forming efficiency (CFE) growth assay, and standard luminescence-based methods (Ceriotti et al., 2007; Xiao et al., 2002; Xing et al., 2005).

Despite the documented success in the EIS measurement of the impedance time series and the derivation of the half-inhibition concentration, the application of EIS for cytotoxicity assessment can go considerably beyond this. As the name implies, it can be used to scan the sample impedance over a wide range of frequencies, typically covering several decades. Another promising and intriguing feature of the EIS assay is the noise analysis of the impedance time series. A few published ECIS results have demonstrated that cell motion may reveal itself as a fluctuation in the measured impedance, which is always associated with living cells and persists even when the cells grow into a confluent layer (Giaever and Keese, 1991; Lo et al., 1993).

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The toxin cytochalasin B interferes with cytoskeleton function by inhibiting actin polymerization (Bonder and Mooseker, 1986; Brown and Spudich, 1981). At sufficiently high concentration, cytochalasin poisoning of cells leads to a number of morphological and functional effects, including arborization, inhibition of endocytosis and secretion, suppression of cytoplasmic division, and enucleation (Carter, 1967; Hirano and Kurimura, 1974; Ohmori et al., 1992). In the present study, ECIS was used to monitor human umbilical vein endothelial cells (HUVEC) in response to the challenge of cytochalasin B, ranging from 0 to 10 μM. By performing both micromotion and frequency scan experiments and analyzing the impedance data, we are able to distinguish cytochalasin B levels as low as 0.1 μM, suggesting that these two methods provide a more sensitive assessment of cytotoxicity than the measurement of time course of overall impedance.

2. Materials and methods

2.1. Cell culture

The human umbilical vein endothelial cells (Cambrex, Walkersville, MD) were cultured at 37 °C and 5% CO_2 in endothelial cell growth medium (EGM; Cambrex) which was supplemented with the following: 10 ng/ml human recombinant epidermal growth factor, 1 μg/ml hydrocortisone, 50 μg/ml getamicin, 50 ng/ml amphotericin B, 12 μg/ml bovine brain extract, and 2% fetal bovine serum (amounts indicate final concentration). Cells were subculture when they were 70% confluent, and the medium was changed every 48 h thereafter. Only HUVECs passaged less than six times were used in experiments.

2.2. Measurement of impedance time course

Electrode arrays, relay bank, lock-in amplifier and software for the ECIS measurement and data analysis were obtained from Applied BioPhysics (Troy, NY). Each electrode array consisted of eight wells which was 1 cm in height and 0.8 cm^2 in bottom area; each well contained a 250 μm diameter gold electrode (area $\sim 5 \times 10^{-4}$ cm^2) and a much larger gold counter electrode. The large electrode and one of the small electrodes were connected via the relay bank to a phase-sensitive lock-in amplifier. A 1 V AC signal at 4 kHz was applied to the sample through a 1 MΩ resistor to maintain an approximately constant current of 1 μA through the sample; i.e., the in-phase voltage was proportional to the resistance, and the out-of-phase voltage was proportional to the capacitive reactance. In all work reported, the electrodes were precoated with 0.2 mg/ml gelatin for 20 min before seeding HUVEC cells. For impedance measurement of the HUVEC monolayer upon addition of cytochalasin B, cells were plated into electrode wells at 10^5 cells/cm^2 density and allowed to attach and spread for at least 24 h before impedance was measured. After 24 h in culture, the confluency and viability of the cell monolayer was confirmed by light microscopy and electrically by the impedance values. Cytochalasin B (Sigma–Aldrich, St. Louis, MO) in DMSO or DMSO alone as a control was added to each cell-covered electrode well. The electrical impedance of each well was measured every 2 min and up to 16 individual wells were followed successively. For detection of cell micromotion, impedance data of each well were taken every second with exquisite sensitivity until 2048 points had been acquired and then another well was measured. The time-series data were normalized and numerically analyzed by calculating power spectrum, variance (the square of the standard deviation), and variance of the increments as we previously described (Lo et al., 1993).

2.3. Frequency scan measurement

Frequency scan is another main method in ECIS and measures impedance of the cell–electrode system as a function of frequency. To quantify the changes in cell morphology, we measured impedance for a cell-free electrode and the same electrode covered with cells at frequencies ranging from 25 Hz to 60 kHz with respective applied voltages ranging from 0.1 to 1 V. By comparing the experimental data of confluent cell layers with the calculated values obtained from cell–electrode model, morphological parameters such as the junctional resistance between the cells (R_b) and the average cell–substrate separation (h) could be determined (Giaever and Keese, 1991). For cells with disk-like shape such as endothelial cells, we used the cell–electrode model with three adjustable parameters, R_b, $\alpha(=r_c(\rho/h)^{1/2})$, and C_m, to fit the experiment data (Giaever and Keese, 1991):

$$\frac{1}{Z_c} = \frac{1}{Z_n}\left[\frac{Z_n}{Z_n+Z_m} + \frac{Z_m/(Z_n+Z_m)}{(\gamma r_c/2)(I_0(\gamma r_c)/I_1(\gamma r_c))+R_b((1/Z_n)+(1/Z_m))}\right], \quad (1)$$

where Z_c is specific impedance (per unit area) of the cell-covered electrode, Z_n the specific impedance of the cell-free electrode, Z_m the specific impedance through both ventral and dorsal cell membranes, r_c the cell radius, I_0 and I_1 the modified Bessel functions of the first kind in order 0 and 1, and

$$\gamma r_c = r_c\sqrt{\frac{\rho}{h}\left(\frac{1}{Z_n}+\frac{1}{Z_m}\right)} = \alpha\sqrt{\left(\frac{1}{Z_n}+\frac{1}{Z_m}\right)}, \quad (2)$$

$$Z_n = S\left(R_n + \frac{1}{i2\pi f C_n}\right), \quad (3)$$

$$Z_m = 2\left(\frac{1}{R_m} + i2\pi f C_m\right)^{-1}, \quad (4)$$

where ρ is resistivity of the cell culture medium, S the electrode area, R_n the measured resistance of the cell-free electrode, f the frequency of the AC signal, C_n the measured capacitance of the cell-free electrode, R_m the specific resistance of the cell membrane, and C_m the specific capacitance of the cell membrane (Lo et al., 1998).

3. Results and discussion

3.1. Effect of cytochalasin B on the time course of overall resistance

The effect of varying cytochalasin B concentration on the overall resistance of the HUVEC monolayer was monitored for 20 h. Sixteen electrodes were followed one after another with each well's data point requiring a few seconds. Although the impedance of each electrode well was set to be measured every 2 min, fluctuations were observed on each curve at different level. The data were first presented as the measured resistance normalized to its value at the start of each run (Fig. 1). At high concentrations of cytochalasin B such as 2.5, 5.0, or 10 μM with cells, a drastic drop of resistance was observed almost immediately following the addition. At low concentrations such as 0, 0.1, 0.5, and 1.0 μM, the resistance drop was less evident. Noise analysis was also applied to characterize the normalized resistance time courses shown in Fig. 1. The variance and the variance of the increments of the data were calculated and plotted against time (Figure S1a and b in the supplement). Here the variance was calculated for the first 60 points of the data (data points 1 through 60) and this was plotted as one point. Next the 60 point data was shifted over one point (data points 2 through 61) and the variance was calculated and plotted. This process was continued until the last 60 points of data was read. To determine the variance of the increments, first all the increments of the successive data were calculated. Their variance was then calculated