In situ electrochemical detection of embryonic stem cell differentiation

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ABSTRACT

Stem cell sensors have emerged as a promising technique to electrochemically monitor the functional status and viability of stem cells. However, efficient electrochemical analysis techniques are required for the development of effective electrochemical stem cell sensors. In the current study, we report a newly developed electrochemical cyclic voltammetry (CV) system to determine the status of mouse embryonic stem (ES) cells. 1-Naphthyl phosphate (1-NP), which was dephosphorylated by alkaline phosphatase into a 1-naphthol on an undifferentiated mouse ES cell, was used as a substrate electrochemically to monitor the differentiation status of mouse ES cells. The peak current in the cyclic voltammetry of 1-NP increased linearly with the concentration of pure 1-NP ($R^2 = 0.9623$). On the other hand, the peak current in the electrochemical responses of 1-NP decreased as the number of undifferentiated ES cells increased. The increased dephosphorylation of 1-NP to 1-naphthol made a decreased electrochemical signal. Non-toxicity of 1-NP was confirmed. In conclusion, the proposed electrochemical analysis system can be applied to an electrical stem cell chip for diagnosis, drug detection and on-site monitoring.

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1. Introduction

Stem cell therapy has the potential to dramatically change the treatment of intractable human diseases such as Parkinson’s disease, ischemic heart diseases, diabetes, and degenerative joint diseases (Lindvall, 2003). The two broad types of stem cells in stem cell therapy are adult stem cells and embryonic stem (ES) cells. Adult stem cells, which are found in adult tissues, repair systems by replenishing the body with specialized cells; however, the number of tissue types these cells can regenerate is limited and do not include several vital organs, such as blood, skin or intestinal tissues. (Hwang et al., 2007) Embryonic stem cells are self-renewal and pluripotent which means that they can differentiate into each of the more than 200 cell types of the adult body, including neurons, cardiomyocytes, hepatocytes, islet cells, skeletal muscle cells and endothelial cells (Coleman et al., 2007; Takahashi et al., 2007).

Cell-based chips hold great promise for applications in cell based detection. These chips must be composed of at least two parts (El-Ali et al., 2006). One part is the microfluidic device for analysis of living cells. For example, polydimethylsiloxane (PDMS) based microfluidic devices are composed of an array of micro injectors integrated in a base flow channel. The microfluidic device controls the injection of drugs into cell cultures. The other part of the chip is the detection apparatus called cell based sensors. In a cell based sensors, living cells act as the detector. Monitoring the condition of living cells is the most critical component of a cell chip (Choi et al., 2005; Kim and Choi, 2007). There are two types of systems for monitoring the conditions of living cells, an optical detection system and electrical (electrochemical) detection system (Yea et al., 2007). Optical detection systems are based on an optical or fluorescent reaction with cells, such as Octamer-4 (Oct-4) immunohistochemistry staining and alkaline phosphatase (AP) staining assay. Optical detection systems have the distinct advantage of being able to visualize changes in cells. However, this type of system prevents miniaturization of the instrument and the optical signals cannot be transformed into electrical signals (Michalet et al., 2005; Singh et al., 2008; Zhu et al., 2007). On the other hand, an electrical detection system allows for the miniaturization of the whole detecting sensor instrument and the cell signals to be easily monitored and analyzed. Despite these advantages, electrical cell detection systems are much less developed than optical detection systems. Living cells have been analyzed as an electrochemically dynamic system with electron generation and electron transfer at the interface of living cells due to redox reactions (Matsunaga and
Namba, 1984). Intact living cells have been investigated in many electrochemical circumstance such as electron transfer at electro active centers in cells, open circuit potential at the cell/sensor interface, electric cell-substance impedance sensing (ECIS), scanning electrochemical microscopy (SECM) to obtain images of the respiratory activity of collagen-embedded living cells, electrochemical impedance spectroscopy (EIS), and the oxygen electrode (Bard et al., 2006; Lu and Gratzl, 1999; Cui et al., 2006; Fasching et al., 2006; Kaya et al., 2003; Kasai et al., 2006a,b; Wolf et al., 2008; Bard et al., 1989) In our previous study, the voltammetric behavior of HeLa cells was determined using a gold electrode as the working electrode in phosphate buffered saline (PBS). This study showed the effect of anti-cancer drugs on a cell chip using the cyclic voltammetry and potential stripping analysis methods (El-Said et al., 2009). However, the differentiation of mouse embryonic stem cells has not been detected with an electrical or electrochemical system.

In this study, we developed an electrochemical cell based sensors to monitor the differentiation status of mouse embryonic stem cells (ES). 1-Naphthyl phosphate (1-NP) has a phosphate containing double benzene ring. The phosphate group of 1-NP is known to be dephosphorylated into 1-naphthol by reacting with alkaline phosphatase, which is one of the embryonic stem cell markers, and 1-NP and 1-naphthol have totally different electrochemical properties. Therefore, in this study, we traced the electrochemical signal of 1-NP, the sensing substrate, to monitor the differentiation of mouse ES cells.

2. Materials and methods

2.1. Materials

1-Naphthyl phosphate was purchased from Sigma–Aldrich (N5602). Phosphate buffered saline (PBS) (pH 7.4, 10 mM) solution was purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of reagent grade.

2.2. Undifferentiated mouse ES cell culture

Mouse embryonic stem cells (J1) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% FBS, 1 mM sodium pyruvate, 1 × 10⁻⁴ M 2-mercaptoethanol, 1 × nonessential amino acids, and leukemia inhibitory factor (LIF) (1000 U/ml) at 37°C and 5% CO₂. The number of cells was determined with a hemacytometer after trypsin blue exclusion.

2.3. Embryoid body (EB) formation and differentiation

EB formation was performed with J1 ES cells as previously described (Abe et al., 1996). ES cells (2 × 10⁶ cells) were seeded onto bacterial-grade Petri dishes in DMEM containing 10% fetal calf serum without LIF. After 2 days of suspension culture, EBs were collected and trypsinized. 1.37 × 10⁶ cells were replated on 0.2% gelatin-coated well plates containing the same medium. The number of cells was counted after 3 days.

2.4. Fabrication of an electrochemical cell

A 2-chamber slide (Lab-Tek™, Nunc, company information), of which the culture area was 4.2 cm²/well, was used as an electrochemical cell in this study. We used CHI 101, CHI 111, and CHI 115 electrodes as the working gold electrode, the Ag/AgCl reference electrode and the platinum counter electrode, respectively. 2 ml of the J1 cells mixed media was transferred into the chamber by infusion of new culture medium. The number of cells in each chamber was determined with a hemacytometer after trypan blue exclusion.

2.5. Electrochemical sensing of mouse ES cells

The cyclic voltammetry experiments were performed using a CHI660A electrochemical system controlled by the electrochemical system software for CHI660A. The three-electrode system was composed of a gold working electrode, a platinum wire counter electrode, and an Ag/AgCl reference electrode. A 1 M concentrated solution of 1-NP was diluted to 1 mM with PBS buffer (10 mM, pH 7.4). The solution buffer contained 1-NP was changed before the detection step. After the detection, the solution was immediately changed to culture media, because 1-NP can be toxic in case long time exposure. The electrical properties of 1-NP reacted with alkaline phosphatase in the mouse ES cells was monitored to determine the differentiation of mouse ES cells (Fig. 1). All electrical sensing was done in PBS buffer. The cyclic voltammetry assay was performed in a 0.6 V to −0.2 V potential window range at 0.1 V/s scan rate, 37°C, and 5% CO₂. All detection steps performed in 1 min.

2.6. Alkaline phosphatase staining immunoassay

Alkaline phosphatase (AP) staining immunoassay kit was purchased from Sigma–Aldrich. First, the cells were fixed with a fixation solution made by mixing 25 ml of citrate, 65 ml of acetone and 8 ml of 37% formaldehyde. The cells were then maintained in the alkaline-dye mixture, which was composed of sodium nitrate, FRV-alkaline solution and naphthol AS-BI alkaline solution, in a dark room for 15 min. Finally, the cells were counterstained with a Hematoxylin solution.

2.7. Western blotting

ES cells and EBs were washed with PBS, collected in RIPA buffer (containing 0.2% Triton X-100, 5 mM EDTA, 1 mM PMSF, 10 mg/ml leupeptin, 10 mg/ml aprotinin) and lysed for 30 min on ice. Protein samples (40 µg) were loaded onto each lane, size-fractioned by polyacrylamide gel electrophoresis, and transferred to a PVDF membrane. After blocking, the membranes were incubated with anti-Oct4 (Millipore, Billerica, MA, USA) and Sox2 (Santa Cruz Biotechnology, Santa Cruz, CA) with 5% BSA in TBST buffer overnight at 4°C. After washing, the membranes were incubated for 1 h at room temperature with an HRP-goat anti-rabbit IgG secondary antibody conjugate and HRP rabbit anti-goat IgG secondary antibody conjugate (1:2000, Zymex). Immunoblots were detected using an ECL western blotting detection system (Amersham International, Little Chalfont, UK) and visualized after exposure to film.

2.8. MTT assay

Tetrazolium dye (MTT; 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was generally used for the determination of cytotoxic effects on the growth and cell viability. The MTT assay was conducted essentially according to the manufacturer’s protocol. Briefly, ES cells were plated in 24-well plates. 1-NP was treated for 1 min. Then, MTT (0.5 mg/ml) was added to each well. Cells were incubated with MTT for 4 h. The insoluble formazan was dissolved in a solubilization solution (0.04 M HCl in absolute isopropanol) at room temperature for 20 min. Cell viability was assessed by measuring the absorbance at 540 nm.

3. Results and discussion

3.1. Detection of 1-naphthyl phosphate

The electrochemical signals at 1-NP concentrations ranging from 0.2 mM to 1 mM were quantitatively determined (Fig. 2). As
shown in Fig. 2a, the peak current increased up to around 0.1 V at high 1-NP concentrations and then slowly shifted to negative voltages as the 1-NP concentration decreased. Fig. 2b shows the linear relationship between the 1-NP peak current and the concentration of 1-NP ($R^2 = 0.9623$). This linear relationship demonstrates the reliability to quantify the concentration of 1-NP using the cyclic voltammetry assay.

3.2. Electrochemical response of undifferentiated mouse ES cells

1-NP is dephosphorylated to 1-naphthol by the AP on undifferentiated mouse ES cells. AP is generally recognized as an embryonic stem cell marker, which means that the differentiated stem cells do not contain an AP. The electrochemical property of 1-NP in the mouse ES cells 15 min post addition of 1-NP is shown in Fig. 3a. The reduction peak current decreased as the number of cells increased.

![Fig. 1. Schematic representation of the electrochemical detection system used to monitor the differentiation of mouse ES cells by enzymatic dephosphorylation of 1-naphthyl phosphate into 1-naphthol.](image1)

![Fig. 2. (a) Cyclic voltammograms at different concentrations of 1-naphthyl phosphate (from 0.2 mM to 1 mM) and a scan rate of 100 mV/s. (b) The reduction peak current increased linearly with 1-NP concentration ($R^2 = 0.9623$). Bars are ±1 standard deviation (N=3).](image2)

![Fig. 3. (a) Electrochemical responses of 1-NP for different numbers of undifferentiated mouse ES cells (from 5000 to 200,000 cells). (b) The reduction peak current decreased linearly as the number of undifferentiated mouse ES cells increased ($R^2 = 0.9623$). Bars are ±1 standard deviation (N=3).](image3)
Fig. 4. (a) Western blotting for Oct-4 and Sox-2 protein in undifferentiated (U-ES cells) and differentiated (D-ES cells) mouse ES cells. (b) Immunocytochemical staining for Oct-4 and Sox-2 protein in undifferentiated mouse ES cells (green channel). Cell nuclei were stained with DAPI (blue). (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

3.3. Confirmation of mouse ES cell differentiation

We used mouse ES cells in electrochemical experiments 2 days post embryoid body formation. However, we could not conclusively determine if the mouse ES cells were differentiated, despite the formation of embryoid body. Therefore, we examined the expression of Oct4 and Sox2 transcription factors, which are two of the main regulators of pluripotency in ES cells and have been used as undifferentiation markers, by western blotting (Takahashi et al., 2007). Fig. 4(a) shows that both the Oct-4 and Sox-2 protein were expressed in undifferentiated cells, but not in differentiated cells. In addition to the western blot analysis, the expression of both proteins in undifferentiated cells was confirmed by immunohistochemistry (Fig. 4(b)). The green channel in Fig. 4(b) indicates the expression of both proteins.

Fig. 5. (a) Effects of 1-NP on the cytotoxicity of undifferentiated mouse ES cells for 1 min exposure. The viability of undifferentiated mouse ES cells was determined by MTT assays. Each data point represents the mean percentage of three independent experiments. Bars are ±1 standard deviation. (b) Alkaline phosphate staining of undifferentiated mouse ES cells and (c) alkaline phosphate staining of differentiated mouse ES cells.
3.4. Cytotoxicity of 1-naphthyl phosphate on mouse ES cells

As described above, we tested whether differentiation of mouse ES cells could be determined by analyzing the electrochemical signals of 1-NP in solution. Before assessing whether this electrochemical signal analysis can be used for the development of a stem cell chip, the toxicity of 1-NP to mouse ES cells should be determined. The effect of 1 mM 1-NP on the cell viability was evaluated by MTT assays. (El-Said et al., 2009) The relative viability was determined to be 99.65% (Fig. 5a). Therefore, the result of this cell viability test clearly demonstrated that 1-NP was non-cytotoxic to mouse ES cells. Fig. 5b and c show the results of AP staining of the 1-NP treated cells. No difference between 1-NP non-treated cells and treated cells were observed in the AP staining experiment. In addition, all of the cells were positive for AP staining. This indicates that the mouse ES cells still have embryonic stem cell properties. Therefore, we can conclude that 1-NP did not affect stem cell differentiation or cell viability.

4. Conclusion

In this study, a new electrochemical analysis technique was developed to monitor differentiation of mouse ES cells with the enzymatic dephosphorylation of 1-NP to 1-naphthol. In the cyclic voltammogram, the peak current decreased linearly with an increase in the number of undifferentiated mouse ES cells; in contrast, the peak current did not change with the number of differentiated mouse ES cells. Therefore, the electrochemical analysis technique developed in this study was able to quantify undifferentiated mouse ES cells using the peak current. This study is based on the enzymatic reaction of alkaline phosphatase. The enzyme is representative cell marker for ES cells, but some other somatic cell lineages also contain it. Besides ES cells, alkaline phosphatase is particularly concentrated in liver, bile duct, kidney and bone. Although the proposed detection method is not perfect to determine the differentiation of ES cells, the method can be a rapid screening tool for ES cells. Thus, this electrochemical detection technique can be applied to the development of a stem cell chip for diagnosis, drug detection and on-site monitoring.

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