Cytotoxicity test based on electrochemical impedance measurement of HepG2 cultured in microfabricated cell chip

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Abstract

This paper presents the use of electrochemical impedance measurement on a cell chip to monitor cell growth as a consequence of treatment with potentially cytotoxic agents. The cell chip consists of an eight-well cell culture chamber incorporated with a three-electrode system on each well. The gold electrode for impedance measurements is fabricated by sputtering on polycarbonate film. Human hepatocellular carcinoma cell (HepG2) is adapted to cytotoxicity test using the cell chip. Although the relatively small quantity of cells on the electrode has been measured indirectly, the cell chip can monitor toxic effects on the HepG2 cells cultured in the cell chip continuously and detect cellular behavior without multiple reagents. The cells in the stationary phase after plating are used for the cytotoxicity experiment and the impedance is decreased after treatments with several toxicants, such as tamoxifen and menadione, indicating the detachment of dead cells. These results reveal that the microfabricated cell chip system provides an easy and real-time monitoring method for cytotoxicity test.

Keywords: Cell-based assay; Cell chip; Cytotoxicity test; Electrochemical impedance; Hepatotoxicity; HepG2

Cell-based assays refer to any experiments based on the use of live cells, which include a variety of methods for measuring cell viability, cytotoxicity, proliferation, motility, and morphology [1–3]. The cell viability assay includes the method measuring the number of live cells, while cytotoxicity assay indicates the number of dead cells. These cell-based assays are frequently used for drug discovery using high-throughput screening (HTS) [4], environmental assessment of chemicals [5], and bio-sensors for monitoring cellular behavior [6]. According to a recent report on drug discovery [2], 30% of the attrition of potentially new drug candidates are due to the failure of toxic verification and the presence of toxic side effects. Therefore, cytotoxicity testing in various cell types has become one of the fundamental tools for drug discovery [1,4]. As an example, hepatotoxicity is a major concern for drug toxicity because most toxic effects are influenced by drug metabolism in hepatocytes, which have been used in most drug discovery and development laboratories [7–10].

Some biochemical methods, such as the MTT assay [8,10], the neutral red uptake assay [5,7], and ATP measurement [11], are widely used in hepatotoxicity screening assays. However, these traditional biochemical methods are time consuming and laborious and require complex steps with multiple reagents at every prescheduled time point. For in vivo experiments they do not give quantitative results without affecting target cells. In addition, it is
difficult to monitor continuous behavior against toxic effects and to analyze the real time change of cell viability. Therefore, many researchers have mainly focused on the development of an alternative method for continuous monitoring of toxic effects [12–19].

Among these methods, an impedance measurement using the microfabricated electrode may provide the ideal method for detecting cellular behavior without multiple reagents [15–17]. Due to the impedance change caused by adherent cells growing on the electrode surface, the relative quantity of cells on the electrode has been measured indirectly. The electric cell-substrate impedance sensing (ECIS) technique was exploited for the attachment and spreading of epithelial MDCK cells on different protein coatings and was used to investigate the influence of divalent cations on spreading kinetics [17]. However, this method shows only the response of cell attachment on the electrode and not various cytotoxic effects on spreading cells. Recently, the ECIS technique was used as the cytotoxicity test for fibroblastic cells [18,19]. A very important factor in the development of the cell-based assay system is obtaining reasonable reproducibility of the measured data because each cell shows different behavior even if the same conditions are applied to the cell culture system [19,20].

In this study, we have developed a microfabricated cell chip with a three-electrode system based on electrochemical impedance measurement. The cell chip system is also applied to monitor cell growth and cytotoxic effects for human hepatocellular carcinoma cells (HepG2). Three-electrode configuration has the advantage that it represents better reproducibility than traditional impedance measurement, which can exactly measure the impedance change under constant voltage by the potentiostat [21,22]. In addition, to the best of our knowledge, use of the impedance technique to monitor cytotoxic effects using hepatoma cells, which is one of the most important cell lines in cytotoxicity testing [7], has not yet been reported. The conditions for the cell chip operation and relationships among the MTT assay, the fluorescence test, and the impedance measurement are discussed herein.

Materials and methods

Microfabrication of the cell chip

The cell chip (70 × 38 mm) consists of an eight-well cell culture chamber incorporated with a three-electrode system, which consists of a working electrode (WE), a reference electrode (RE), and a counter electrode (CE) on each well (Fig. 1). All electrodes were fabricated by sputtering gold on polycarbonate film (SK Anitech, Korea) with a thickness of 350 μm. A metal shadow mask was used to create the electrode patterns by sputtering.

To find an optimum electrode size, three different active areas (with diameters of 250 μm, 500 μm, and 1 mm) of working electrode were formed by screen printing of insulation paste (adhesive PD955PY; Heraeus, Germany) on the gold-sputtered electrode. The insulation layer was cured for 10 min at 120 °C. An eight-well polystyrene chamber (Lab-Tek II chamber slides; Nunc, Denmark) for cell culture was adhered to the polycarbonate film using silicon adhesive paste (World Precision Instruments, FL). The assembled cell chip was treated by air plasma (200 mTorr, 200 W) for 20 s to make a sterilized environment. An expanded plasma cleaner (Harrick Science, Ossing, NY) was used to induce air plasma. This treatment also facilitates the attachments of the HepG2 cells on the surface of the cell chip due to the hydrophilicity of the plasma-treated surface. The culture area of each well on the cell chip was 0.7 cm². To connect the signal wire of three electrodes with the impedance analyzer, the PCB was assembled with the cell chip using a Leeno Pin (Leeno Industrial, Korea).

Cell culture

The human hepatocellular carcinoma cell (ATCC HB8065) was chosen for the toxicology experiment and applied to the cell chip for monitoring hepatotoxicity. The cell layers were briefly rinsed with phosphate-buffered saline (PBS) with pH 7.4 (Gibco, Grand Island,
NY). Then, trypsin–EDTA solution (0.25% trypsin and 1 mM EDTA · 4Na; Gibco) was used to detach the cells and Dulbecco’s modified Eagle’s medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) was supplemented to the dispersed cell layer. The cell cultures were maintained at 37 °C under 5% CO₂ in a humidified water-jacketed incubator [23].

Unless otherwise noted, appropriate aliquots of the cell suspension were refreshed and diluted to suitable concentrations of 5 × 10⁶ cells ml⁻¹. Then, 600 μl of diluted cells and media in the plate was transferred into each well of the cell chip. The same concentration of cells was transferred and subcultured in a 96-well plate. These cells grown on the 96-well plate were equally treated with toxicants to compare the results of the cell chip system. The cell number was counted by a hemacytometer (Marienfeld, Germany).

**Impedance measurement and data analysis**

An impedance analyzer that includes eight potentiostats, a channel selection logic system, and a lock-in amplifier was specially designed by Analog Research System (Pohang, Korea). The measurement system has expansion units up to 128 channels for future high-throughput applications. To maintain a constant working electrode potential using a feedback control circuit, eight potentiostats were used to simultaneously control individual wells incorporated with a three-electrode system. Each of the signals selected from the potentiostats was coupled to the lock-in amplifier, and the impedance was detected. The resistance component of the impedance was calculated by a previously reported [22,24] method. A channel selection logic system was used to orderly detect the impedance signals of the eight potentiostats. The overall system was controlled by a personnel computer through an RS232 interface. The impedance was measured under the condition that an oscillating potential of 10 μV was applied to each well through working and reference electrodes. The measurement current should be reduced to 10 nA for minimizing the current effect on HepG2 cells. To find an optimum condition for impedance measurement, the impedance measurements were performed in the frequency range 10 Hz–100 kHz. The data sampling time for impedance measurement was every 10 min and each well of the eight-well cell culture chamber was scanned in 30-s intervals.

**Cytotoxicity test in the cell chip**

In the cytotoxicity experiment using the cell chip, cells in the stationary phase after plating were used. The initial concentration of cells inoculated on the cell chip was 5 × 10⁶ cells ml⁻¹. After treatments with several toxicants, the impedance was monitored during 4–6 h until the cells were completely affected by the specific toxicant. As cytotoxic agents, tamoxifen and menadione (Sigma–Aldrich) were used. Tamoxifen was dissolved in dimethyl sulfoxide (DMSO; Sigma) for each experiment and added to the cell chip at concentrations between 150 and 400 μM [8]. Similarly, menadione was added to the cell chip at concentrations between 100 and 400 μM [9]. Triton X-100 concentrations between 0.01 and 0.06% (v/v) were also used to compare the toxicity equivalent to 100% cell death.

**Fluorescence test in the cell chip**

After the cytotoxicity experiment using the cell chip, 500 μl of working solution (Live/Dead Viability/Cytotoxicity Kit; Molecular Probes, OR) containing 2 μM calcein AM and 4 μM ethidium homodimer-1 (EthD-1) was added directly to the cultured cells in each well of the cell chip, so that all cells were reacted with the solution. The cells were incubated for 45 min to prevent contamination or sample drying at room temperature. Then, PBS was added to wash out the working solution in the cell chip. After detaching the eight-well cell culture chamber, the fluorescence labeled cells in the surface of the polycarbonate film of the cell chip were observed under a fluorescence microscope (Epi-fluorescence equipment; Carl Zeiss, Germany). It was confirmed that green fluorescence caused by calcein AM represents live cells and red fluorescence caused by EthD-1 represents dead cells [25].

**MTT assay**

For comparison with a conventional MTT (Sigma) assay, cells in DMEM-based incubation media at a density of 5 × 10⁶ cells ml⁻¹ were loaded at 50 μl per well in a 96-well plate. Then 50 μl of toxicants dissolved in 1% DMSO (Sigma) was added per well and the plate was placed in a humidified incubator at 37 °C under 5% CO₂ for 8 h. Then 15 μl of 5 mg ml⁻¹ MTT with 0.9% NaCl solution was added to each well and the cells were incubated for an additional 3 h. Then 150 μl of isopropanol (Sigma) with 0.4 N HCl was added to each well and the plate was shaken on an orbital shaker at room temperature for an additional 12 h. Finally, the optical density of each sample was measured at 570 nm [10].

**Results and discussion**

**Optimization for impedance measurements**

In the present study, we used a three-electrode configuration (working, counter, and reference electrodes) to measure the impedance, thus providing better reproducibility than traditional impedance measurement. Because a two-electrode system for impedance measurement has
potential variation in the reference electrode which also acts as a counter electrode, a reference electrode must maintain a stable potential by making its area ideally four to five times larger than that of the working electrode [26]. It was reported that there was a significant difference between the sample impedance and the impedance indicated by the ECIS system [18]. These drawbacks of a two-electrode system can be avoided by use of a three-electrode system. We use a potentiostat to control the working electrode potential and investigate the effect of electrode size and working frequency to find an optimum condition for impedance measurement of HepG2 cultured in the cell chip system.

Since the area of the electrodes’ surface, voltage, and current inevitably affect the status of cells, optimum conditions for impedance measurement were investigated prior to detecting cellular behavior without multiple reagents. For the selection of an optimum working electrode size among the electrodes with diameters of 250 µm, 500 µm, and 1 mm, two tests were performed in the frequency range 10 Hz–100 kHz. One measured the impedance change between cell and medium and the other measured the reproducibility of data obtained under the same conditions. HepG2 cells cultured overnight were used in the experiments. As shown in Fig. 2, the impedance signal decreases as the frequency increases. For smaller gold electrodes, higher impedance signals are obtained. This result is the same as that of the ECIS system, which uses small gold electrodes with diameters of 250 µm deposited on the bottom of cell culture dishes [17]. However, the impedance signals from the working electrode with a diameter of 250 µm show unstable responses relative to those of 500 µm and 1 mm, resulting in poor reproducibility in the impedance measurement experiments. This is the result of nonuniformity due to the fabrication process. Because the active area of the working electrode was determined by the printing of the insulation paste using a 325-µm mesh screen, the smaller-sized electrodes were not as uniformly formed as those of 500 µm and 1 mm. Thus, this leads to poor reproducibility of the electrode response. Moreover, cellular behavior in the working electrode with a diameter of 250 µm did not reflect the representative toxic effect on the cells because the relatively small quantity of cells on the electrode attached only in this area unlike those on the 500-µm and 1-mm electrodes. Therefore, we select the working electrode with a diameter of 500 µm in view of sensitivity and reproducibility because the larger electrode with a diameter of 1 mm shows lower sensitivity.

In addition, at the lower frequencies, there were no significant differences in the impedance change in cells and medium compared with the higher frequencies of more than 10 kHz (Fig. 2). Differences between impedance values of cells and medium are almost the same at the frequency of 10 kHz, regardless of the electrode size tested. In most experiments, we use the working frequency of 10 kHz for providing sensitive impedance measurement for cell growth monitoring. Also the data sampling time for impedance measurement was set every 10 min to minimize the damage of cells due to the electrical stress [27].

**Impedance change as the cell growth of HepG2 cultured in the cell chip**

To observe the relationship between the cell concentration and the impedance, HepG2 cells diluted to suitable concentrations from $1 \times 10^5$ to $4 \times 10^7$ cells ml$^{-1}$ were cultured on the cell chip overnight. Then, the impedance was measured at 10 kHz. As cells begin to attach and cover the surface of the electrode the impedance value increases. Fig. 3 shows that the impedance increases as the concentration of inoculated HepG2 cell increases. Independent experiments using different cell chips were also compared with the data obtained from each well in the same cell chip. For interbatch experiments using different cell chips, the coefficient of variation is more than 10% (chip-to-chip variations) while for intrabatch experiments in the same cell chip it is 4–8% (well-to-well variations). Therefore, the data obtained from each well in the same cell chip were compared following cell growth and cytotoxicity experiments.

**Fig. 2.** Effect of frequency on the impedance changes in the cell chip. Electrodes with diameters of 250 µm, 500 µm, and 1 mm were used to measure the impedance difference between cells and medium. HepG2 cells cultured on the cell chip overnight were used in the experiments.

**Fig. 3.** Impedance change as the cell growth of HepG2 cultured in the cell chip. Cell images (×200) on the gold electrode in the microfabricated cell chip are also consistent with the HepG2 cell growth curve. It is shown that the impedance graph (Fig. 4) represents lag (2), log (3), stationary (4), and death (5) phases as a typical cell growth curve. The data were plotted using the average value of...

The impedance signals obtained from six wells of the cell chip. From these results, it would be possible to monitor cell growth by the cell chip more conveniently and continuously. At the stationary phase (4) as shown in Fig. 4, the deviation of the impedance signals from six wells in the cell chip was the lowest. It seems that the cells dispersed over the entire electrode area to form a fully confluent monolayer. At this point, the impedance signal was very stable for several hours regardless of the cell-cultured wells. Therefore, the cells in the stationary phase after plating were used for the cytotoxicity experiment. However, at the death phase (5), the impedance signal was seriously variable during the experiments, indicating the individual characteristics of the cells even with the use of the same seed culture [19,20]. It seems that the variations result from the different physiological phenomena (such as a tendency to form clumps rather than a suspension of individual cells) in each cell-cultured well in the cell chip. For most cell-based assays, the variations are more likely to be caused by the cells rather than by the assay chemistry [1].

**Cytotoxicity test based on the impedance measurement in the cell chip**

From the relationship between the impedance and the cell growth curve, it is possible to measure live or dead cells by detecting the impedance change. Cells were allowed to grow to confluence and were then used in carried out the experiments of treatment with potentially cytotoxic agents. After obtaining the stable impedance signal of stationary phase, the change of the impedance was monitored to investigate the cytotoxicity on the HepG2 cultured in the cell chip. In this study, various concentrations of toxicant such as tamoxifen and menadione were used to detect live and/or dead cells by toxicants. Tamoxifen induces apoptosis in human malignant cell lines, especially in hepatocytes [8]. Menadione produces superoxide radicals and induces apoptosis [9]. As shown in Fig. 5, the impedance of the medium was the lowest and was almost constant over the course of the experiments. For the control experiment (without treatment of toxicant), we used a fresh medium instead of the toxicant. A little

![Fig. 3. Impedance change during cell attachment experiments. After HepG2 cells diluted to suitable concentrations were cultured on the cell chip overnight, the impedance was measured at 10 kHz. The concentration of cells inoculated on the cell chip was varied from 1 \times 10^5 to 4 \times 10^7 cells ml^{-1}. The points shown are the mean of at least four intrabatch experiments.](image)

![Fig. 4. HepG2 cell growth curve by impedance measurement in the cell chip. The data from six wells in the cell chip were averaged and are displayed as the mean and standard deviation. Cell images (×200) on the gold electrode in the microfabricated cell chip were consistent with the HepG2 cell growth curve: (1) medium, (2) lag, (3) exponential, (4) stationary, and (5) death phases.](image)
change was shown when the medium was slightly changed by the addition of fresh medium. However, there is a spike in impedance after the addition of toxicant solution. This increase appeared to be dose dependent with tamoxifen in Fig. 5A and with menadione in Fig. 5B, while absent in cells with no toxicants added. It seems that the spike is a temporary effect before the high concentration of toxicants diffused equivalently. At 30 min after the injection of the toxicants, the impedance of control cells dropped to the previous value and was stabilized. However, as the concentration of the two toxicants increased the impedance decreased. This is the reason that the cells were detached from the gold electrode surface and were severely damaged. These results are compared with 0.06% Triton X-100 treatment, indicating the same toxicity equivalent to 100% cell death (Fig. 5C).

We also investigated the cell morphology using conventional fluorescent assays after the impedance measurement. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity determined by the enzymatic conversion of virtually nonfluorescent cell-permeable calcein AM to intensely fluorescent calcein. It is well known that the polyanionic dye calcein is well retained within live cells, producing intense uniform green fluorescence (extinction/emission = ~495 nm/~515 nm). EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing bright red fluorescence in the dead cells (extinction/emission = ~495 nm/~635 nm). The determination of cell viability depends on these physical and biochemical properties of cells. However, cytotoxic events that do not affect these cell properties may not be accurately assessed using this method. It is shown that, as the concentration of toxicants increases, the intensity of red light always becomes stronger and that of green light becomes weaker (data not shown). These results imply that the impedance measured by the cell chip was consistent with the intensity of fluorescence and that the cell chip could be useful for cytotoxicity experiments.

In addition, to confirm the relationship with a traditional assay, the MTT assay using the same toxicant concentrations was performed. The results of the MTT assay are consistent with cytotoxicity tests by the cell chip. As shown in Fig. 6, there are relationships between the MTT assay and the impedance signal in the cell chip, which implies that the impedance measured by the cell chip can produce results similar to those that the MTT assay produces. In the MTT assay, active mitochondrial dehydrogenase of living cells converts the yellowish MTT to an insoluble purple formazan. This conversion does not take place in dead cells. This water-insoluble formazan can be soluble in isopropanol, and then the dissolved material can be measured spectrophotometrically at 540 nm [10]. Since the MTT assay measures cell viability based only on mitochondrial activity, cytotoxic agents for the MTT assay were limited to the specific compounds that affect mitochondria. However, the cell chip can monitor toxic effects in the cells continuously and detect cellular behavior without multiple reagents. Therefore, the impedance measurement method based on the cell chip system could replace the conventional MTT assay.
Conclusion

The impedance measurement technique is considered a very useful candidate because it can be applied to many quantitative, automated, and HTS experiments. In this study, the microfabricated cell chip based on the impedance measuring system was designed and fabricated. The HepG2 cultured in the cell chip was also applied to cytotoxicity tests including cell growth monitoring experiments. The results described in this paper reveal that the cell chip system would provide an easy and real-time monitoring method for cytotoxicity tests, especially for in vitro drug tests. Furthermore, this system could replace many conventional experiments dealing with not only animal cells but also bacterial cells.

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