Correlation between cell migration and reactive oxygen species under electric field stimulation

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Cell migration is an essential process involved in the development and maintenance of multicellular organisms. Electric fields (EFs) are one of the many physical and chemical factors known to affect cell migration, a phenomenon termed electrotaxis or galvanotaxis. In this paper, a microfluidics chip was developed to study the migration of cells under different electrical and chemical stimuli. This chip is capable of providing four different strengths of EFs in combination with two different chemicals via one simple set of agar salt bridges and Ag/AgCl electrodes. NIH 3T3 fibroblasts were seeded inside this chip to study their migration and reactive oxygen species (ROS) production in response to different EF strengths and the presence of β -lapachone. We found that both the EF and β -lapachone level increased the cell migration rate and the production of ROS in an EF-strength-dependent manner. A strong linear correlation between the cell migration rate and the amount of intracellular ROS suggests that ROS are an intermediate product by which EF and β -lapachone enhance cell migration. Moreover, an anti-oxidant, α -tocopherol, was found to quench the production of ROS, resulting in a decrease in the migration rate. © 2015 AIP Publishing LLC.

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INTRODUCTION

Collective cell migration is an important cellular event, which is involved in many different physiological processes such as embryonic development,^{1–3} tissue repair,^{4–6} angiogenesis,^{7–9} and wound healing.^{10–12} In particular, it has been suggested that this collective behavior plays important roles in the invasion and spread of malignant cells.^{13–15} Many physical and chemical factors affect the direction and rate of cell migration. For example, chemotaxis, electrotaxis, and mechanotaxis are phenomena describing how cells respond to certain chemicals, electric fields (EFs), and mechanical cues, respectively, in their environment. Experiments on the chemotaxis of various cells have been widely conducted using both conventional devices such as the Boyden assay¹⁶ and microfluidic devices such as polydimethylsiloxane (PDMS)-based chips.^{17,18} In addition, these phenomena are crucial in cell/tissue development^{19,20} and cancer metastasis.^{21,22} Mechanotaxis, which refers to directional cell migration under fluidic shear stress and substrate stiffness, has recently drawn increasing interest^{23–25} because it impacts stem cell differentiation,²⁶ disease progression,²⁷ and wound healing.²⁸

Electrotaxis or galvanotaxis is the directional migration of adherent cells or organisms in response to direct current (dc) or alternating current (ac) EFs.^{29–31} Various cells including cancer cells,^{32,33} stem cells,^{34–36} and epithelial cells^{37–39} migrate collectively under dcEFs. *Caenorhabditis elegans* also migrates from the anode to the cathode at a specific angle

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proportional to the strength of an EF.⁴⁰ Furthermore, *C. elegans* migrated at different deflecting angles under the same EF at different developmental stages.⁴¹ Some cells migrate in the direction of an EF, while others move in the opposite direction of the field.^{32,42} Electric signals may play a crucial role in the metastasis of cancer cells by enhancing their migration.^{43,44} These physiological signals, so-called transepithelial potentials, originate from the differential distribution of ion channels on polarized cells.³² Another important phenomenon related to electrotaxis is the wound-healing process. When a wound occurs, an endogenous lateral EF of approximately 40–200 mV/mm is generated nearby.⁴⁵ By sensing and orienting themselves toward this field, the cells are able to migrate in a specific direction to repair the damage.^{46,47} Therefore, exogenous electrical stimuli (dcEFs and acEFs) have been widely applied to accelerate wound healing in animal and human models.^{48–50} Despite the fact that endogenous and exogenous EFs have been shown to increase wound-healing rates, clarifying the underlying mechanism of these effects requires further study.

Microfluidic devices have recently become powerful tools for providing cells with controllable microenvironments that best mimic *in vivo* biological matrices. Such devices have been applied to study the electrotaxis of cells and wound-healing processes under well-controlled exogenous EFs.^{33,51,52} For example, Sun *et al.* reported a new method to incorporate a 3D scaffold with electrotaxis measurement in a microfluidic device.³² In that study, the electrotactic response of lung cancer cells in 3D foam scaffolds, which resemble the *in vivo* pulmonary alveoli, was investigated. The results showed that in traditional 2D petri dishes and sophisticated 3D scaffolds, the migration direction was the same, but there were clear differences in cell morphology, directedness, and migration rates. Another microfluidic electrical stimulated wound-healing chip was designed and fabricated to integrate an EF with a modified barrier assay.⁵³ The migration of fibroblasts was studied under different conditions, such as in serum, under various EFs, and in the presence of wound-healing promoting drugs.

Here, a modified microfluidic chip based on our previous designs was fabricated to study the correlation between cell migration and applied EFs in NIH 3T3 fibroblasts.^{33,52} We attempted to correlate first the electrical stimuli with the production of reactive oxygen species (ROS) and then the produced ROS with the migration rate and directedness. ROS are chemically reactive molecules. Exposure to a high concentration of ROS might cause cell structure damage, aging, and even cell death.⁵⁴ In contrast, a low concentration of ROS is believed to be an important signal in cell proliferation, migration, gene expression, enhancement of signal transduction, and oxidative stress response.^{54–56} Superoxide mediates directional cell migration through the AKT, a serine/threonine kinase that has a critical regulatory role in diverse cellular processes, and extracellular-signal-regulated kinase (ERK) signaling pathways under applied EFs.^{57,58} Moreover, the effects of β -lapachone, a chemical known to promote the proliferation of fibroblasts in the wound-healing processes, on the production of ROS and migration rates under EFs were studied. Passive correlations between the applied EF and the migration rate, between the applied EF and the production of ROS, between the addition of β -lapachone and the migration rate, and between the addition of β -lapachone and the production of ROS were identified. In addition, an anti-oxidant, α -tocopherol, was shown to quench the production of ROS and decrease the migration rate. These results could enhance our understanding of the mechanisms of electrotaxis and wound healing.

MATERIALS AND METHODS

Chip design and fabrication

The design of the microfluidic chip is shown in Fig. 1. The pattern was drawn in AutoCAD (Autodesk) and then loaded into a CO₂ laser scriber (ILS2, Laser Tools & Technics Corp.) to ablate the desired patterns on polymethylmethacrylate (PMMA) substrates.^{59–61} Four layers (Fig. 1(a), thickness = 1 mm) of PMMA sheets were thermally bonded together to form the electrical stimulation part, which was then attached to a cover glass via a patterned double-sided tape (8018, 3M; thickness = 0.07 mm), which served as the cell culture areas (Fig. 1(b)). The top layer had two medium inlets, two outlets, and two holes for electrodes. The widths of the bottom four culture areas (two copies) were 4 mm, 2.07 mm, 4.14 mm, and 8.28 mm. The

FIG. 1. Design of the microfluidic chip. (a) Four layers of polymethylmethacrylate (PMMA) were bound together and then attached onto a culture dish via double-sided tape. (b) The integrated chip thus formed.

4 mm-width area served as the negative control without EF, while the others were EF-stimulated regions (Fig. 1(b)). The cytotoxicity of PMMA substrates and double-sided tape on cells was examined, and no significant change in cell viability was observed.³³

EF calculation and simulation

The direct current required to generate the desired EFs inside the culture areas was calculated on the basis of Ohm's law. The strength of the EF through a bulk material is $E = I/(\sigma A_{eff})$, where I is the applied dc, σ is the electrical conductivity of the material, and A_{eff} is the effective cross-sectional area of the material. Using $\sigma = 1.38 \Omega^{-1} \text{ m}^{-1}$ for Dulbecco's modified Eagle medium (DMEM) and $A_{eff} = 0.1449 \text{ mm}^2$ (width = 2.07 mm; height = 0.07 mm), 0.2898 mm^2 (width = 4.14 mm; height = 0.07 mm), or 0.5796 mm^2 (width = 8.28 mm; height = 0.07 mm) in the EF-stimulated area, the EF strength is calculated to be $I \text{ (A)} \times 5 \times 10^6 \text{ mV/mm}$, $I \text{ (A)} \times 2.5 \times 10^6 \text{ mV/mm}$, or $I \text{ (A)} \times 1.25 \times 10^6 \text{ mV/mm}$, respectively, under a constant applied current I . The numerical simulation of the EF inside culture areas was performed using the commercial software COMSOL Multiphysics (COMSOL).

Cell preparation

The fibroblast cell line NIH 3T3 was purchased from the Bioresource Collection and Research Center (BCRC), Taiwan. A complete medium consisting of DMEM (Gibco) and 10%

calf serum (CS, Invitrogen) was used for the cell culture. The cells were incubated in tissue culture poly-styrene flasks (corning) in 5% CO₂ at 37 °C until 90% confluence before seeding into the microfluidic chip.

Experimental procedure

The microfluidic chip was assembled inside a laminar flow hood. The inlets were connected to two syringes driven by syringe pumps (NE-300, New Era). Phosphate buffered saline (PBS) buffer (1×) was channeled into the chip and kept within the channel overnight under 5% CO₂ at 37 °C. Then, the syringes were pushed gently by hand to remove bubbles, and 1 × PBS was replaced with DMEM. A total of 10⁶ cells suspended in 1 ml culture medium (DMEM with 10% CS) were injected into the chip. For temperature control during the experiments, the chip was mounted on top of a transparent indium tin oxide (ITO) glass (Part No. 300739, Merck), which was connected to a proportional-integral-derivative controller (TTM-J4-R-AB, JETEC Electronics Co.), for maintaining the temperature at 37 ± 0.5 °C via feedback from a thermal coupler (TPK-02 A, TECPEL) clamped tightly between the ITO heater and the chip. The chip-heater unit was then mounted onto a microscope stage with fresh culture medium continuously pumped into the chip at a flow rate of 20 μl/h overnight. The next day, culture medium with and without the addition of 1 μM β-lapachone or 20 μg/ml α-tocopherol were pumped into the two culture channels (each had four culture areas) at 20 μl/min for the first 20 min. The flow rate was then slowed down to 20 μl/h. During the same period, different strengths of EFs were introduced to the cells by connecting the Ag/AgCl electrodes to a dc power supply (GWInstek, Taiwan) set at the constant-current model. The setup of the agar salt bridge network has previously been described in detail.⁵²

The intracellular ROS level was measured using the fluorescence-based indicator 2'-7'-dichlorodihydrofluoresce diacetate (2'-7'-DCFDA) (Sigma, USA). This fluorophore has excitation and emission wavelengths of approximately 488 nm and 524 nm, respectively. DCFDA is non-fluorescent as it passively diffuses into the cells. To make it fluorescent, the molecule must become de-acetylated and oxidized. After EF and/or β-lapachone stimuli, cultured cells inside the microfluidic chip were incubated with DCFDA (5 μM in DMEM) for 20 min at a flow rate of 20 μl/min and another 20 min at a flow rate of 20 μl/h. Thereafter, the chip was washed with DMEM for 20 min, and fluorescent images of different field of views (FOVs) were captured using an inverted fluorescent microscope (TS-100, Nikon) equipped with a CCD camera (DS-Qi1, Nikon).

Data analysis

We observed the cell migration inside each culture area using a bright-field inverted microscope (CKX41, Olympus). This microscope was programmed to automatically repeat the observation in a certain time interval. For each area, photos were taken every 15 min for 2 h for tracking cell migration. The cell migration path and directedness were tracked by ImageJ software (National Institute of Health). The migration rate in μm/h is defined as the average displacement of cell migration per hour. The migration directedness is defined as the average cosine θ , $(\sum_i \cos \theta_i)/n$, where θ is the angle between the vector of the applied EF (from positive to negative) and the vector from the start to the end position of a cell, and n is the total number of cells considered. A directedness of +1 indicated that all cells migrated toward the cathode, while a -1 value indicates that all cells migrated toward the anode. The directedness of a group of randomly moving cells should be close to 0. In the ROS quantification, the fluorescent intensity of the cells was analyzed again by ImageJ software. For each FOV, a polygon was drawn to enclose each cell, and the mean intensity within the area was calculated. The background intensity was then subtracted to obtain the absolute signal. For each experimental condition, at least 80 cells were selected from at least 3 independent experiments for data analysis. The standard error of the mean (SEM) was also calculated.

RESULTS AND DISCUSSION

EF calculation and simulation

As mentioned earlier, an endogenous EF of approximately 40–200 mV/mm is generated near a wound. According to Ohm's law (see **Materials and Methods**), an applied dc of $80\ \mu\text{A}$ would generate EF strengths of 400 mV/mm, 200 mV/mm, and 100 mV/mm in the three EF stimulated areas, respectively. Fig. 2(a) shows the degree of simulation of the EF inside the culture areas. The EF strengths were almost uniform inside each area and close to the calculated values. Fig. 2(b) shows the profile of EF strengths across the middle of one culture channel. The average values were 382 mV/mm, 191 mV/mm, and 101 mV/mm in the three EF stimulated areas, respectively, indicating errors of $<5\%$ between the calculations and simulations.

Correlation between the EF and cell migration

Cell migration was observed and imaged using an inverted microscope with a $4\times$ objective lens and a digital SLR (single-lens reflex) camera (60D, Canon). There were a total of eight areas to be imaged, with four different EF strengths for two different chemicals. A motorized stage controlled by a customized MATLAB GUI program was used to locate each area repeatedly at an interval of 15 min for 2 h. The FOV, about $1\text{ mm} \times 1\text{ mm}$, was selected to be in the center of each area. Cells moved out of focus or FOV were not selected for analysis. Scatter plots indicating cellular distributions after 2-hr EF and β -lapachone stimuli were shown in Fig S1 in the supplementary material.⁷⁸ The migration rates of NIH 3T3 fibroblasts under different strengths of EFs are shown in Fig. 3(a). Without the addition of β -lapachone, the migration rate

FIG. 2. (a) The degree of stimulation of the electric fields (EFs) inside the microfluidic chip. (b) The profile of EF strength across the middle of one culture channel.

increased in response to increasing EF. The mean rates were $10.5 \mu\text{m/h}$, $17.3 \mu\text{m/h}$, $24.5 \mu\text{m/h}$, and $31.7 \mu\text{m/h}$ for an EF of 0 mV/mm , 100 mV/mm , 200 mV/mm , and 400 mV/mm , respectively. These values are close to those observed in other studies. For instance, Brown *et al.* showed that under an EF of 400 mV/mm , NIH 3T3 fibroblasts generally migrated at rates from $10\text{--}20 \mu\text{m/h}$.⁶² In addition, in the wound-healing assay reported by Sun *et al.*, the wound-healing rate of NIH 3T3 cells was $14 \mu\text{m/h}$ under an EF of 150 mV/mm .⁵³ With the addition of $1 \mu\text{M}$ β -lapachone, the migration rates were higher compared to those without β -lapachone, and these values again increased with increasing EF. For example, the mean rates increased from $17.3 \mu\text{m/h}$ to $22.9 \mu\text{m/h}$ under 100 mV/mm , and from $31.7 \mu\text{m/h}$ to $38.4 \mu\text{m/h}$ under 400 mV/mm in response to the addition of β -lapachone. A low concentration of β -lapachone accelerated the proliferation and migration of NIH 3T3 fibroblasts via ERK and p38 signaling.⁶³ However, in our previous studies, β -lapachone alone or a low concentration of β -lapachone combined with an EF increased the wound-healing rate, but an overdose of β -lapachone in the presence of EF instead decreased the rate.⁵³ This difference could be due to the fact that in the previous study, cell-cell interactions in the wound-healing assay were important, but in the present case, cells were more or less free of contact from other cells.

The migration directedness defined in $\cos\theta$ is shown in Fig. 3(b). In the absence of EF, NIH 3T3 fibroblasts did not show prominent directional movement (directedness = $0.03\text{--}0.13$). Under an EF but without β -lapachone, these cells migrated toward the cathode, giving a mean migration directedness of 0.57 , 0.83 , and 0.91 for EFs of 100 mV/mm , 200 mV/mm , and 400 mV/mm , respectively. Brown *et al.* reported that the mean directedness of NIH 3T3 fibroblasts exposed to a 400 mV/mm EF for 1 h was 0.726 , compared to -0.02 for the same cells before EF stimuli.⁶² As shown in the figure, this directedness was not affected by the presence of β -lapachone. Orientation of cells in response to EF and β -lapachone was shown in Fig S2 in the supplementary material.⁷⁸ By comparing box plots of cells under different stimuli, we observed that cells began to orientate themselves perpendicularly to the applied EF at

FIG. 3. The migration rate (a) and directedness (in $\cos\theta$) (b) of NIH 3T3 fibroblasts after being stimulated by different strengths of EFs with and without the addition of $1 \mu\text{M}$ β -lapachone.

400 mV/mm. No significant differences were observed after adding β -lapachone. We conclude that this chemical increased the migration rate but not the directedness and cell orientation, while EF enhanced the rate, directedness, and cell alignment.

CORRELATION BETWEEN THE APPLIED EF AND THE PRODUCTION OF ROS

ROS are an important indicator when cells are subject to environmental stresses such as UV, heat exposure, or EFs. ROS regulate certain cellular processes including the epithelial-mesenchymal transition,^{64,65} epithelial-stromal metabolic coupling,⁶⁶ and angiogenesis.⁶⁷ Therefore, we suggested that an EF increases the production of ROS, which in turn accelerates cell migration. Fig. 4(a) shows fluorescent images of NIH 3T3 cells under different strengths of EFs with and without the addition of β -lapachone. The mean fluorescent intensity with SEM in an arbitrary unit is plotted in Fig. 4(b). Without β -lapachone, the mean intensities were 15.7, 33.7, 43.3, and 66.1 for EFs of 0 mV/mm, 100 mV/mm, 200 mV/mm, and 400 mV/mm, respectively. This result suggests that EF stimulates the production of ROS in a strength-dependent manner. The higher the strength of the EF, the more ROS were produced. The same trend was observed when β -lapachone was added. The production of ROS increased with an increasing

FIG. 4. (a) Fluorescent and bright-field images of NIH 3T3 fibroblasts after being stimulated by different strengths of EFs with and without the addition of 1 μ M β -lapachone. Scale bar = 25 μ m. (b) Mean fluorescent intensities of cells in (a).

EF and with the addition of β -lapachone. For example, the mean fluorescent intensities increased from 33.7 to 55.8 under 100 mV/mm, from 43.3 to 66.4 under 200 mV/mm, and from 66.1 to 94.7 under 400 mV/mm. The correlation between the migration rate (Fig. 3(a)) and the production of ROS (Fig. 4(b)) is shown in Fig. 5. A R^2 of 0.95 indicates that there is a strong linear correlation between these two variables.

To further demonstrate that ROS is the key factor inducing cell migration, we attempted to quench the production of ROS with an anti-oxidant, α -tocopherol. We stained the cells with DCFDA to measure the production of ROS under EF stimuli. It was observed that with a treatment of 20 μ g/ml α -tocopherol, the fluorescent intensities per cell did decrease under all EF strengths (Figs. 6(a) and 6(b)). The corresponding scatter plots indicating cellular distributions were shown in Fig. S3 in the supplementary material.⁷⁸ Under no or low (100 mV/mm) applied EFs, α -tocopherol did not affect the cell migration rate (Fig. 6(c)). When increasing the EFs to 200 and 400 mV/mm, the addition of α -tocopherol significantly decreased the migration rate, from 16.3 to 13.9 and 23.4 to 18.4 μ m/h, respectively. However, the migration directedness did not change in response to adding α -tocopherol (Fig. 6(d)). We also quantified the orientation of cells in response to EF and α -tocopherol and found that cell began to align perpendicularly to the applied EF at 400 mV/mm. (Fig. S4 in the supplementary material).⁷⁸ Similarly, α -tocopherol did not affect cell alignment. Again, we conclude that ROS is one of the important factors impacting the migration rate under EF stimulation.

DISCUSSION

In this study, we have shown that there is a strong correlation between the production of ROS and the migration rate under EF stimulation. When ROS were produced by the addition of β -lapachone or quenched by the addition of α -tocopherol, the migration rate also increased or decreased, respectively. EF stimuli increase the production of ROS in NIH 3T3 fibroblasts and other cells.^{68–72} Serena *et al.* explored the effects of EF stimulation on ROS generation and cardiogenesis in embryoid bodies.⁷¹ In that study, the authors concluded that EF stimulation plays a role in cardiac differentiation of human embryonic stem cells through mechanisms associated with the production of endogenous ROS. Li *et al.* showed that dcEF directed the migration of HT-1080 fibrosarcoma cells to the cathode and stimulated the production of ROS via the activation of the ERK pathway.⁵⁸ These results explain what we have observed, i.e., an EF stimulated

FIG. 5. Linear correlation between the cell migration rate (Fig. 3(a)) and the production of ROS (Fig. 4(b)).

FIG. 6. (a) Fluorescent and bright-field images of NIH 3T3 fibroblasts after stimulation with different strengths of EFs with and without the addition of 20 $\mu\text{g/ml}$ α -tocopherol. Scale bar = 25 μm . (b) Mean fluorescent intensities of cells in (a). The migration rate (c) and directedness (in $\cos\theta$) (d) of NIH 3T3 fibroblasts after being stimulated by different strengths of EFs with and without the addition of 20 $\mu\text{g/ml}$ α -tocopherol.

cell migration and the generation of ROS in a strength-dependent manner. Considering the effect of β -lapachone, Shiah *et al.* demonstrated a pronounced elevation of ROS in human leukemia HL-60 cells treated with this chemical, through the activation of the c-Jun N-terminal kinase (JNK) pathway.⁷³ In addition, Park *et al.* showed that β -lapachone induced ROS production and the resultant autophagic cell death of U87 MG glioma cells.⁷⁴ ROS are usually thought to be toxic byproducts of cellular metabolism. They have recently been shown to play essential roles in cell proliferation, migration, and adhesion.^{75,76} A low concentration of ROS promotes cell-cell communication and cell proliferation.⁷⁷ Therefore, these studies, in addition to our results, suggest that both EFs and β -lapachone increase the production of ROS, which subsequently enhance cell migration. Moreover, these two stimuli have a synergistic effect on ROS generation and cell migration.

CONCLUSION

In this study, a microfluidic chip was designed and fabricated for studying the effects of an EF and β -lapachone on cell migration and the production of ROS. Four different strengths of EFs with and without the addition of β -lapachone were applied on NIH 3T3 fibroblasts. The cell migration rate increased with increasing EF in a strength-dependent way. Moreover, the addition of β -lapachone further accelerated cell migration. These findings were correlated with the generation of intracellular ROS. Both EFs and β -lapachone stimulated the production of ROS again in an EF-strength-dependent manner. We found that there is a strong linear correlation between the cell migration rate and amount of ROS produced. We thus conclude that both EF and β -lapachone increase the production of ROS, which in turn accelerate cell migration. While the addition of α -tocopherol quenches the production of ROS, this also decreases the migration rate. Thus, our chip provides an easy and fast platform for *in vitro* studies of cellular behavior under EF and chemical stimuli in a high-throughput setting.

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