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Cell-based high-throughput proliferation and cytotoxicity assays for screening traditional Chinese herbal medicines

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ABSTRACT

A simple, reliable, high-throughput screening method was developed and used to assess the pharmaceutical effects of extracts of traditional Chinese herbal medicines (TCHMs). This method is based on 3-dimensional (3-D) cultures of mouse embryonic stem (mES) and human colon cancer and breast cancer cells expressing enhanced green fluorescent protein (EGFP) in polyethylene terephthalate (PET) fibrous scaffolds on modified 384-well plates with online monitoring of culture fluorescence for dynamic responses of cells to drugs present in culture media. Cell responses to deoxycholic acid and the extracts of 3 TCHMs (*Ganoderma lucidum* spores, *Ginkgo biloba*, and *Epimedium brevicornum*) at various concentrations were investigated for their effects on proliferation and cytotoxicity. The screening results, i.e., the growth responses of cancer cells to those drugs, were consistent with what have been reported in the literature, confirming the reliability of the new screening approach. Different from previous screening methods for both TCHMs and western medicines that used animal models or 2-D cell-based assays with single cell lines, this 3-D cell-based screening method employs both cancer and normal cells and thereby provides a way for quick, direct evaluation of the anticancer effects of TCHMs. This method also offers assessment on the side effects of TCHMs.

1. Introduction

In recent years, cell-based assays have played increasingly important roles in drug discovery because cells can provide more representative responses to drugs than molecular assays and are easier to use in a high-throughput format than animals [1–3]. A cell-based assay is a procedure for testing or measuring the activity of a drug or biochemical on a cell sample through assessing specific parameters or responses (e.g., cell number, viability, proliferation, or cytotoxicity) of cells. Extensive research efforts have been made to the application and improvement of cell-based assays for drug screening and drug discovery [4]. To reduce drug screening and development costs, cell-based assays is moving towards automation, miniaturization, and high throughput via the combination of microfabrication, microfluidics, molecular biology, cell culture techniques, and precise measuring instruments [3,5]. For instance, Zhang et al. developed a perfusion platform for sandwich-cultured hepatocytes for screening drugs that induce hepatotoxicity [6]. However, this method needs to disrupt cell culture and collect cells for viability and hepatotoxicity measurements. Online measurements of dissolved oxygen, pH, and conductivity were developed as a non-invasive method to monitor cell growth continuously [7,8]. However, these indirect measurements were unable to accurately quantify cell number and proliferation rate and therefore often failed to accurately determine the cytotoxicity effects of the investigated drugs. Fluorescence-based detection techniques are becoming increasingly prevalent due to its high detection sensitivity and the industry-wide move away from using radioisotopes [9]. The anticancer activities of some compounds isolated from two medicinal herbs were identified by measuring fluorescence resonance energy transfer in the apoptosis of cancer cells [10]. However, cells were cultured in conventional tissue culture plates and the method required invasive operations for the fluorescence measurement.

Recently, a 3-dimensional (3-D) cell culture system expressing the enhanced green fluorescent protein (EGFP) was developed for high-throughput drug discovery [1]. This system was successfully used in evaluating the cytotoxicity of dexamethasone on embryonic stem cells and the dosage effects of 5-fluorouracil and gemcitabine on colon cancer cells. In the present study, we further explored the application of this 3-D cell-based fluorescence assay for high-throughput screening of TCHM extracts for anticancer activities. Numerous chemical compounds have been isolated from TCHMs

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and exhibited treatment effects for a variety of cancers. For examples, a water-soluble glucan isolated from the spores of *G. lucidum* demonstrated anti-tumor activities on lung cancer during a two-week treatment on mice [11]. Kaempferol and quercetin isolated from *G. biloba* showed apoptotic effects on oral cavity cancer cells [12] and suppressed the growth of human colon cancer cells [13]. Icaritin and estrogenic prenylflavone extracted from *E. brevicornum* by methanol inhibited the growth of breast cancer cells [14,15]. TCHMs have a great potential in cancer treatment and are generally less expensive than pure chemicals. However, TCHMs as a valuable source for new anticancer drugs have not been fully exploited due to the lack of a reliable and high-throughput screening method.

In this study, human colon cancer HT-29, breast cancer MCF-7, and mouse embryonic stem (mES) cells expressing EGFP were used as target cells for the screening of potential anticancer activities and cytotoxicity of TCHM extracts. Most cell-based drug screening methods use only a single cell line [10,16], which gives incomplete evaluations on the drug effects and often missed undesirable or detrimental effects to normal cells such as embryonic stem cells. To demonstrate the feasibility and advantages of our 3-D cell-based fluorescence assay, anticancer and cytotoxicity effects of 3 commonly used TCHMs (*Ganoderma lucidum* spores, *Ginkgo biloba*, and *Epimedium brevicornum*) were evaluated. These TCHMs were selected because they have been shown to have treatment effects on various cancers, allowing us to compare our screening results and validate the 3-D culture fluorescent assays conducted in the modified 384-well plates in a high throughput fashion. In addition, we also compared and validated the assay results with those obtained in conventional 2-D cultures in commercial 48-well plates and 3-D cultures in spinner flasks using the manual cell counting method. This is the first study applying the 3-D culture system with online high-sensitivity fluorescence measurement for high-throughput screening of TCHMs.

2. Materials and methods

2.1. Preparation of herbal medicine extracts

Spore powders of *G. lucidum* (also called Lingzhi) and dried leaves of *G. biloba* and *E. brevicornum* were purchased from Cai Zhi Lin (Guangzhou, China), a drugstore of traditional Chinese herbal medicines. The extract powders of *G. biloba* and *E. brevicornum* were prepared from their dried leaves. Briefly, dried leaves (1 g) were cut into small pieces and added to distilled water (100 ml) in a round-bottom flask (500 ml) equipped with a condenser and refluxed at 120 °C for 30 min. The resulting solution was centrifuged at 4,000 rpm for 10 min. The supernatant was freeze-dried at −40 °C for 24 h. These dried extract powder were stored at −80 °C.

2.2. Cultures and media

Murine embryonic stem (mES) D3 cells (ATCC CRL-1934), human breast cancer MCF-7 cells (ATCC HTB-22), and human colon cancer HT-29 cells (ATCC HTB-38) expressing EGFP were obtained by transfection with the plasmid PEGFP-N3 (Clontech, Palo Alto, CA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfection and establishment of these stable EGFP-expressing cell lines have been described elsewhere [1,17]. The expression of EGFP in these cells was under the control of a strong constitutive promoter, the human cytomegalovirus (CMV) promoter, which gave a high expression level of the fluorescence protein proportional to the viable cell number, allowing the use of EGFP fluorescence to monitor and quantify cell growth.

Unless otherwise noted, the mES cells were cultured in ES growth medium in gelatin-precoated T-flasks, and were passaged every 3 days to maintain their undifferentiated state. The ES growth medium was prepared by supplementing the knockout Dulbecco's modified Eagle's medium (DMEM, Gibco®, Invitrogen Corp., Carlsbad, CA) with 10% (v/v) fetal bovine serum (FBS, Invitrogen), 50 U/ml penicillin (Invitrogen), 50 μg/ml streptomycin (Invitrogen), 0.1 mM non-essential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 μM β-mercaptoethanol (Sigma–Aldrich Corp., St. Louis, MO), and 100 μM leukemia inhibitory factor (LIF, Cat. # ESG1106, Millipore, Billerica, MA). The MCF-7 and HT-29 cells were cultured in DMEM (Invitrogen) plus 5% (v/v) FBS in T-flasks without gelatin coating. All T-flask cultures were incubated at 37 °C with 5% CO_2 in a water jacketed CO_2 incubator (Forma Series II, Thermo Electron Corp., Waltham, MA). Cells in T-flasks were harvested by trypsination and used in drug screening studies described below.

For screening the effects of TCHMs on ES and cancer cells in multiwell plates, powders of *G. lucidum* spore, and *G. biloba* and *E. brevicornum* extracts were dissolved in the respective growth media described before to various concentrations (w/v) (0%, 0.01%, 0.1%, and 1.0%). The media with TCHM extracts were then sterilized by filtering through sterile polyvinylidene fluoride (PVDF) syringe filters (0.22 μm pore size, Fisher Scientific, Pittsburgh, PA). Media containing deoxycholic acid (DCA, Sigma–Aldrich Corp., St. Louis, MO) at various concentrations (0.1, 1, and 10 mM) were also used as a positive control in testing cell responses to drugs in the 3-D cultures.

2.3. Screening of TCHMs in 3-D cultures

Screening of TCHMs was performed with cells cultured in polyethylene terephthalate (PET) fibrous scaffolds on a modified 384-well plate, which contained 40 micro-bioreactors, each was made of 9 wells with their walls removed except for four pillars remaining for holding a piece of the fibrous disk (diameter: 3.9 mm) as the cell scaffold placed in the center well, following the procedures described elsewhere [1,17]. Briefly, after soaking in the TCHM-containing media for 12 h, each PET scaffold was seeded with 4,000 mES cells or 6,000 MCF-7 or HT-29 cells on a 96-well plate. After 1-h incubation for cell attachment, the seeded PET scaffolds were washed with media to remove unattached and loosely attached cells, and then transferred to a modified 384-well plate with 900 μl media in each micro-bioreactor. The 384-well plate was incubated in a 5% CO_2 incubator at 37 °C, and cell growth was monitored by measuring the culture fluorescence. At least triplicate micro-bioreactors were used for each condition tested.

2.4. Effects of G. lucidum spore in 2-D cultures

For comparison, 2-D cultures of mES, MCF-7 and HT-29 cells were also studied in 48-well plates (Corning®, Lowell, MA) with media with or without 0.01% (w/v) *G. lucidum* spore (GLS). Each well containing 500 μl media was seeded with approx. 90,000 mES cells, 40,000 MCF-7 cells, or 50,000 HT-29 cells and incubated in a water-jacketed CO_2 incubator at 37 °C. Cells in each well were harvested at predetermined times and quantified by counting the cell number using a haemacytometer (Superior, Marienfeld, Germany). Triplicate wells were used for each sampling point.

2.5. 3-D static and dynamic cultures

For comparison and validation of the fluorescent assay results, mES cells were also cultured in 3-D PET scaffolds in static 24-well plate (Corning®, Lowell, MA) and dynamic spinner-flask cultures to study the effect of GLS on cell proliferation. For the static culture, each PET scaffold (1.5 cm in diameter) was seeded with approx. 4,600 cells in 100 μl media, incubated for 1 h at 37 °C for cell attachment, washed with media to remove unattached and weakly attached cells, and then transferred to a new well containing 1 ml fresh media. For the spinner-flask culture, a 25-ml spinner flask with a PET scaffold (1.5 cm × 11 cm) fixed around the flask wall was seeded with ~2 million cells in 12 ml media and agitated with a stirring bar at 80 rpm for cell attachment to the scaffold [18]. After seeding, both the 24-well plate and spinner flask were incubated in a CO_2 incubator at 37 °C, with periodically refreshing the media, for ~6 days.

2.6. Analytical methods

Unless otherwise noted, cell proliferation in 3-D cultures was monitored by measuring the EGFP fluorescence with a microplate reader (GENios Pro™, Tecan, Männedorf, Switzerland) at the bottom reading mode, with the excitation and emission wavelengths of 485 nm and 535 nm, respectively. For 2-D cultures, cell number was measured by Trypan blue exclusion method using a haemacytometer (Superior, Marienfeld, Germany). For the spinner flask cultures, the final cell number was estimated using the nuclei counting method described elsewhere [19]. Briefly, the PET matrix with cells was taken out of the spinner flask at the end of culturing period and incubated in the nuclei counting solution (0.1 M citric acid, 0.1% (w/v) crystal violet) at 37 °C for 24 h. The matrix was then vigorously vortexed to release cell nuclei, which were counted under a microscope. The concentrations of glucose and lactate in culture media were measured by using YSI Biochemistry Select Analyzer (YSI Life Science, Yellow Spring, OH).

2.7. Statistical analysis

Unless otherwise noted, results are shown as mean values ± SE ($n \geq 3$) and Student's t-test was performed with $p < 0.05$ as statistically significant.

3. Results and discussion

3.1. Screening of TCHMs in 3-D cultures

The growth kinetics of mES, HT-29 colon cancer, and MCF-7 breast cancer cells in media containing various TCHMs were