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Human neuroblastoma (SH-SY5Y) cell culture and differentiation in 3-D collagen hydrogels for cell-based biosensing

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Abstract

Cell-based three-dimensional systems are desirable in the field of high throughput screening assays due to their potential similarity to in vivo environment. We have used SH-SY5Y human neuroblastoma cells cultured in 3-D collagen hydrogel, confocal microscopy and immunofluorescence staining, to assess the merit of the system as a functional, cell-based biosensor. Our results show differences between 2-D and 3-D resting membrane potential development profile upon differentiation. There was no statistically significant difference in SH-SY5Y proliferation rate between 2-D monolayer and 3-D collagen culture formats. A large percentage of cells (2-D, 91.30% and 3-D, 84.93%) did not develop resting membrane potential value equal to or lower than −40 mV; instead cells exhibited a heterogeneous resting membrane potential distribution. In response to high K^+ (50 mM) depolarization, 3-D cells were less responsive in terms of increase in intracellular Ca^{2+}, in comparison to 2-D cells, supporting the hypothesis that 2-D cell calcium dynamics may be exaggerated. L-Type Ca^{2+} expression levels based on staining results was inconsistent with Bay K 8644 channel activation results, strongly suggesting that either the majority of the channels were non-functional or could not be activated by Bay K 8644. In general, the results in this study confirm the depolarization-induced differences in intracellular calcium release when cultured using a 2-D versus a 3-D matrix.

Keywords: Collagen hydrogel; SH-SY5Y; Voltage gated calcium channels; Resting membrane potential; Confocal microscopy

1. Introduction

Voltage gated ion channels are emerging as essential drug targets in the pharmaceutical industry due to their impact on diseases of the central nervous and cardiovascular systems (Denyer et al., 1998; Gonzalez et al., 1999). With the current advance in genomics and combinatorial chemistry, it is now possible to screen thousands of compounds against voltage gated ion channel targets in a 96- or 384-well plate in a short time (Denyer et al., 1998). However, in vitro biochemical assays target specific enzymes or proteins associated with the ion channel and as a result the "hits" generated from such screens often fail when tested in the natural and complex environment of an organism (O'Connor et al., 2000a; Durick and Negulescu, 2001). Thus, in order to produce physiologically meaningful results, it is essential to study the ion channels in living cells (Gonzalez et al., 1999; O'Connor et al., 2000a; Durick and Negulescu, 2001).

Cell-based biosensors prove advantageous because they provide a cell with all of the necessary biological interactions that are available to a cell in its in vivo environment. The biosensors can be utilized to study biological and chemical warfare agents, environmental toxins, or to detect possible deleterious effects of a drug before clinical trials (O'Connor et al., 2000a; Durick and Negulescu, 2001). Many cell-based biosensors in use today are based on a flat, two-dimensional glass or plastic surface that may not produce results characteristic of in vivo (Cukierman et al., 2002; O'Connor et al., 2000a). Extracellular matrix (ECM) is the three-dimensional substrata, which provides a direct interaction between cells via integrin receptors in vivo. These ECM mediated receptor

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cell interactions are responsible for the regulation of cell proliferation, migration, and adhesion (Friedl and Brocker, 2000). Recent studies have shown that performing cell-based assays in three-dimensional substrates may provide physiologically relevant results (Cukierman et al., 2001; Friedl and Brocker, 2000). Thus, many three-dimensional substrates are being considered to address the need to study cell behavior in an "in vivo" environment. Examples of 3-D substrates include microgravity bioreactors (Jessup et al., 1993), electrospun nanofibers (Matthews et al., 2002), micro- and nano-structured substrata (Powers et al., 2002), and natural and synthetic scaffolds (Sherwood et al., 2002; Deng et al., 2002).

Collagen hydrogel has been used in various three-dimensional cell behavior studies (Cukierman et al., 2001; O'Connor et al., 2000b). In a previous paper, Mao and Kisaalita (2004a) studied voltage gated calcium channel (VGCC) properties of the neuroblastoma cell line, IMR-32 in a 3-D collagen hydrogel, and concluded that the optical and mechanical properties of collagen hydrogel (0.5–1.0 mg/ml collagen) are suitable for a cell-based biosensor. Furthermore, Mao and Kisaalita (2004a,b) showed a significant difference in calcium response to high K^+ depolarization between cells grown in 2-D flat dishes (monolayer) versus 3-D hydrogel. Day 13 cells (differentiated) in 3-D collagen gel showed a calcium response, however, Day 2 cells (undifferentiated) did not. Unfortunately, IMR-32 cells do not develop a resting membrane potential (V_m) characteristic of nerve cells (Rao and Kisaalita, 2001). Also, Mao and Kisaalita (2004a) comparative proliferation results were not conclusive. The purpose of this paper is to extend the study to a human neuroblastoma cell line that develops a neuronal-like resting membrane potential, and to compare cellular proliferation between the traditional 2-D and 3-D collagen hydrogel.

2. Materials and methods

2.1. Cell line and cell culture

SH-SY5Y cell line was chosen because it has been shown to develop a more characteristic resting membrane potential (Sonnier et al., 2000) and to possess voltage gated calcium channels upon differentiation (Morton et al., 1992; Reuveny and Narahashi, 1993; Seward and Henderson, 1990; Reeve et al., 1995). Also, it has a short doubling time and it is capable of differentiating into different neuron-like subtypes. SH-SY5Y cell line was cultured in 75-cm^2 tissue culture flasks (Costar, Cambridge, MA) with 30 ml growth medium at 37 °C in a 10% CO_2 humidified atmosphere. Growth medium was made with minimum essential medium with 10% heat inactivated fetal bovine serum (FBS), 2.2 g/l sodium bicarbonate, and 2 mM L-glutamine (Mao and Kisaalita, 2004a). Growth medium was replaced every other day and cells were passed at 75% confluence.

2.1.1. 2-D culture

For monolayer or 2-D culture, cells were plated on No. 1.5 glass coverslip embedded in a 35-mm petri dish (MatTek Co., Ashland, MA) at a density of 5×10^5 cells per plate. The cells were allowed to grow for 2 days, and then growth medium was replaced with differentiation medium made of 5% FBS, 2.2 g/l sodium bicarbonate, 2 mM L-glutamine, and 200 nM TPA or 1 mM dibutyryl cAMP and 2.5 μM 5-bromodoexyuridine (Mao and Kisaalita, 2004a). The differentiation medium was changed daily.

2.1.2. 3-D collagen-cell culture

Twelve milligrams collagen (Rat tail tendon, Type I, Sigma, St. Louis, MO) was dissolved in 2.5 ml 0.2% (v/v) acetic acid and sterilized under UV overnight in a biosafety hood. The next day, 2.5 ml 2× PBS, 35 μl 1 M NaOH, and 7 ml 1× PBS was added to the solution to obtain a final collagen solution of 1 mg/ml at pH 7.4, which was stored at 4 °C to avoid gel formation (Mao and Kisaalita, 2004a). SH-SY5Y cells at a density of 1.2×10^6 cells/ml were mixed with 1 ml collagen solution and spread on a 35-mm petri dish with an embedded No. 1.5 glass coverslip. The plates were incubated at 37 °C for 2 h to allow gel formation, and then 2 ml growth media was added to the plates. The growth medium was replaced with differentiation medium after 2 days and replaced daily thereafter.

2.2. Proliferation

Cell proliferation was measured in both 2-D and 3-D over 72 h. For 2-D cultures, 500,000 cells were plated in 3 wells each of a 6-well plate and 3 ml growth media was added to each well. Cells were allowed to grow for 24 h after which they were dislodged mechanically, centrifuged, and re-suspended in growth media. Cells were counted using a hemocytometer. This procedure was followed after 48 and 72 h. For 3-D cultures, 1.2×10^6 cells were plated in 35 mm petri plates each according to the procedure described above for 3-D cultures. Cells were released using 3 mg of collagenase Type I (Sigma) dissolved in 1 ml of DMEM. Cells were counted as described above and data was fitted to the growth curve below (Eq. (1)):

$$Y = Ae^{kt} \tag{1}$$

where k is the growth rate, A the number of cells at the start of the experiment and Y is the number of cells at time, t.

2.3. Resting membrane potential (V_m) development

We have previously used a voltage-sensitive dye, oxonol (Kisaalita and Bowen, 1997), to assess the resting membrane potential of cells; unfortunately, it was not possible to perform measurements at the single cell level. In this study, we have used a confocal microscopy method used to determine resting membrane potential, at the single cell level that was previously described by Mao and Kisaalita (2004b) in detail.