A Noncontact Co-Culture Model of Peripheral Neural Cells for Nanotoxicity, Tissue Engineering and Pathophysiological Studies

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ABSTRACT

Cell culture models *in vitro* have long served as tools for the elucidation of cellular and molecular mechanisms of diseases. Recently these versatile models have gained wide acceptance in toxicology and tissue engineering research. Although these models are not exactly the same as the models *in vivo*, they facilitate mechanistic insights pertaining to a particular cell type. Nevertheless, co-cultures of two different but functionally complementary cell types provide structural and functional perspectives single-cell-type models do not. We have initiated a systematic development of this model type employing dorsal root ganglion (DRG) neurons and Schwann cells. Our first model involves non-contact co-cultures of Schwann cells and DRG neurons employing the hanging cell culture insert. The results from our studies to date demonstrate that our non-contact co-culture model is suitable for both high throughput and mechanistic studies in nanotoxicological and pathophysiological research.

Keywords: Co-culture model, DRG neurons, Schwann cells, nanotoxicology, tissue engineering, pathophysiology.

1 INTRODUCTION

The aim of our studies is to systematically develop and characterize cell culture models of neural cells of the peripheral nervous system *in vitro* for nanotoxicological and pathophysiological research. Once such models are developed, they may have wide applications in tissue engineering research. The need for such models is derived from the fact that the peripheral nervous system is most susceptible for the development of neuropathy. Some examples of peripheral neuropathies include drug-(chemotherapy) induced neuropathy [1], disease-induced neuropathy (e.g., diabetes) [2], immune and inflammatory neuropathies, inherited and genetic neuropathies, and other neuropathies induced by toxicity.

The applications of nanomaterials, including nanoparticles, have been increasing exponentially in the last decade and have infiltrated diverse fields. Once they enter the body by inhalation or other routes, nanoparticles may be toxic to one or more organ systems [3]. However, the toxicity of nanoparticles in mammals, especially humans, has not been systematically studied although there is increasing concern about the environmental and health

impact of exposure to nanoparticles of different types [4]. Consequently, we have systematically initiated a series of studies to elucidate the putative cytotoxicity of nanoparticles in various mammalian cell types [4]. For example, once recent study of ours noted that several metal oxide (including titanium and zinc oxides) nanoparticles exert differential cytotoxic effects on human neural cells in cell model systems *in vitro* [4].

There have been, however, extremely few cell model systems developed employing peripheral neural cell types for nanotoxicological and tissue engineering studies. We have therefore initiated a systematic development of this type of models employing Schwann cells and dorsal root ganglion (DRG) neurons. The first model we have developed involved a non-contact co-culture model of Schwann cells and DRG neurons using the hanging cell culture insert. Thus, this type of co-culture cell systems, consisting of two distinctly different but functionally complementary cell types, provides structural and functional perspectives that single-cell-type models do not.

We hypothesized that non-contact co-culture model of Schwann cells and DRG neurons can be useful in elucidating the putative toxic effects of various nanoparticles on the peripheral nervous system. Furthermore, the putative cytotoxic effects of nanoparticles could also influence/disrupt the cell-to-cell communications between the two cell types. To test our hypothesis, we have initially characterized the Schwann cells the DRG neurons in monotypic or single-cell-type cultures. In our ongoing studies, employing our new co-culture, non-contact model, we have recently demonstrated that the survival of DRG neurons is increased when co-cultured with Schwann cells in this construct. In this study, we compare the putative cytotoxic effects of SiO_2 and TiO_2 nanoparticles on dorsal root ganglion (DRG) neurons and Schwann cells employing established cytotoxicity testing [5-7]. Furthermore, we are investigating the putative toxic effect of these nanoparticles on interaction(s) between DRG neurons and Schwann cells in non-contact co-culture.

2 MATERIALS AND METHODS

2.1 Cells and Culture Conditions

Silicon dioxide (SiO_2) nanoparticles (STREM Chemicals, Newburyport, MA, USA; Cat. #93-1434; <12 nm particle size, 99+ % (flumed colloidal silica)) and TiO_2, anatase, nanoparticles (Sigma-Aldrich, St. Louis, MO, USA; Cat. #637254; nanopowder, <25 nm particle size, 99.7% (metals basis)) were dispersed in 100 mL of sterile saline in a sealed conical flask and the suspension stirred at ambient temperature overnight before being employed to be diluted to the specified concentrations for treatment of cells [4]. The immortalized DRG neurons were a gift from Dr. Ahmet Hoke's Laboratory at Johns Hopkins University (Baltimore, MD, USA) [8]. The rat Schwann cell line was obtained from ATCC (Manassas, VA, USA). These cells and their co-culture were cultured in DMEM (Sigma; St Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) sodium pyruvate, 0.292 g/l L-glutamine, 1.5 g/l sodium bicarbonate, 1% (v/v) anti-mycotic and various specified concentrations (5-45 mM) of glucose**.**

2.2 Co-culture Model System

DRG neurons were cultured on a Corning Costar 24-well plate. Schwann cells were then seeded on a Millipore hanging cell culture insert (pore size 0.4 µm) on a separate plate and then introduced to a DRG neuron-containing well after 4 hours to achieve the co-culture condition [7]. DRG neurons (co-cultured with Schwann cells) were exposed to various concentrations of SiO_2 nanoparticles for specified periods and their survival determined employing the cell survival assay.

2.3 Cell Survival (MTT) Assay

DRG neurons or Schwann cells were seeded per well in a 48-well plate in DMEM with specified concentrations of SiO_2 nanoparticles. After incubation at 37^{o}C for 24 or 48 hours, 50 µl of MTT (5 mg/ml in PBS) reagent was added to each well. After incubation for another 4 hours, the medium was removed gently and the cellular reaction product was solubilized in 200 µl DMSO. Then the optical density of the contents of each well was measured in a plate reader at 570 nm [4]. The absorbance corresponds to live cells present in each well [4].

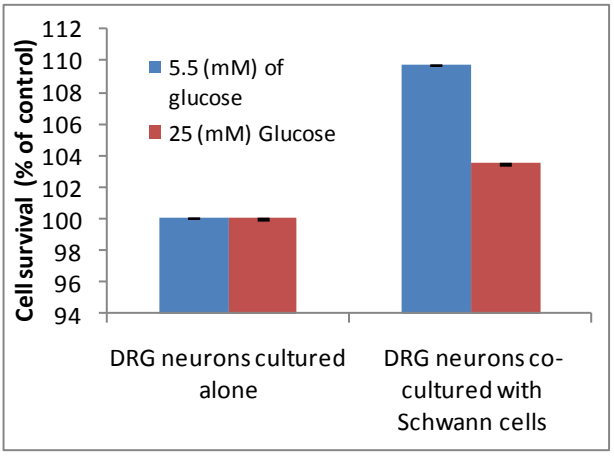

Figure 1: Co-cultures of DRG neurons and Schwann cells using hanging cell culture inserts (non-contact co-culture). Values are mean ± SEM of 6-8 determinations and replicate experiments show the same trend. DRG neurons cells treated in 5.5 mM and 25 mM of glucose for 24 hours are marked with blue color and red color respectively.

2.4 Cellular morphology

DRG neurons, Schwann cells and their co-culture was treated with SiO_2 specified concentration for 24 and 48 hours at 37^0 C as described above and bright field images were acquired by using a Leica light microscope (Leica DM IRB, Bannockburn, IL, USA) equipped with a digital camera (Leica DFC 300FX) [4].

2.5 Statistical analysis of results

Statistical significance of experimental results was analyzed with one-way ANOVA followed by Dunnett's post-hoc test with a minimum significance level set at $p< 0.05$ using the SPSS 17 software package.

3 RESULTS AND DISCUSSION

We are the first to employ dorsal root ganglion (DRG) neurons and Schwann cells to develop co-culture models *in vitro* because DRG neurons are the most susceptible nerve cells to developing pathologies in peripheral neuropathy. We have characterized the two cell types individually with respect to their optimum glucose requirement and activities of enzymes important in modulation of glutamate-glutamine cycling (e.g., glutamine synthetase) that is part of the metabolic and functional inter-dependence between

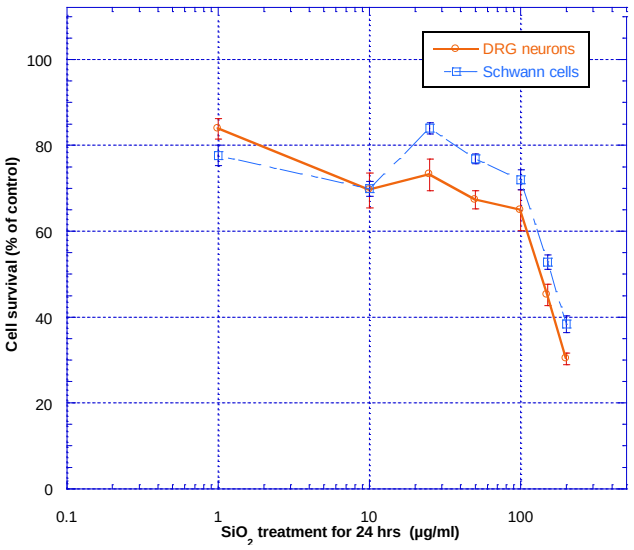

Figure 2: Effects of SiO_2 nanoparticles on DRG neurons and Schwann cells. Values are mean ± SEM of 12 determinations and replicate experiments show the same trend. DRG neurons and Schwann cells treated with SiO_2 nanoparticles for 24 hours are marked red and blue, respectively.

DRG neurons and Schwann cells known to occur *in vivo* (data not shown). We have also studied the protein expression of various apoptosis-related proteins such as BCL-2, BCL-XL, BAX and cytohrome C in the two cell types (data not shown).

To our knowledge, we are the first to determine, in this study, the putative cytotoxic effects of SiO_2 and TiO_2 nanoparticles on neural cells of the peripheral nervous system (i.e., DRG neurons and Schwann cells).

As shown in Figure 2, treatment of DRG neurons or Schwann cells for 24 hours with SiO_2 induced a dose-related decrease in survival of those cells when we progressively increased the concentrations of the nanopartcles from 1 to 200 µM, with IC_{50} values of ~150 µM (Figure 2). At the highest concentration employed, less than 40% of the cells survived (Figure 2).

Because we had previously noted that TiO_2 nanoparticles exerted differential cytotoxic effects on central nervous system neural cells [4], we have investigated the possibility that these nanoparticles may also exert cytotoxic effects on neural cells from the peripheral nervous system. As shown in Figure 3, our on-going studies indicate that treatment with TiO_2 nanoparticles in the dose range so far employed (0.1 to 20 µM) appeared to lower the survival of both DRG neurons and Schwann cells: the effects seemed somewhat dose-related. Moreover, the Schwann cells appeared to be more susceptible to the effect of TiO_2 nanoparticles, suggesting they may exert differential effects on the neural cells of the peripheral nervous system.

We have also employed light microscopy to examine the effects of nanoparticles on DRG neurons and Schwann cells and assess the effects of SiO_2 and TiO_2 nanoparticles thereon (data not shown). In general, the photomicrographs revealed that treatment with the nanparticles induced dose-related changes in cellular morphology consistent with the nanoparticle-induced progressive decrease in cell viability (Figures 2 and 3).

As we have previous noted, treatment with TiO_2 nanoparticles induced dose-related increases in cell death of human central neural cells, marked by apoptosis, and necrosis, and an as yet uncharacterized cell death mechanism that appeared to be a mixture of apoptosis and necrosis [4]. Thus, the results of our ongoing studies with the peripheral nervous system neural cells such as DRG neurons and Schwann cells appear to show some similarity to those we obtained with central neural cells [4]. The DRG neurons and Schwann cells appear also to be susceptible to the cytotoxic effects of SiO_2 and TiO_2 nanoparticles. As we have indicated earlier, we are also determining the cell death mechanisms underlying the cytotoxic effects of SiO_2 and TiO_2 nanoparticles on DRG neurons and Schwann cells. Clearly, this is an important area that merits further study.

4 CONCLUSIONS

Thus, the results from our studies to date as well as those from our ongoing studies demonstrate that our non-contact co-culture model is highly suitable for both high throughput and mechanistic studies in nanotoxicological and tissue engineering research. Moreover, this model is also relevant for the investigation of mechanistic issues associated with peripheral diabetic neuropathy and other disease states of the peripheral nervous system.

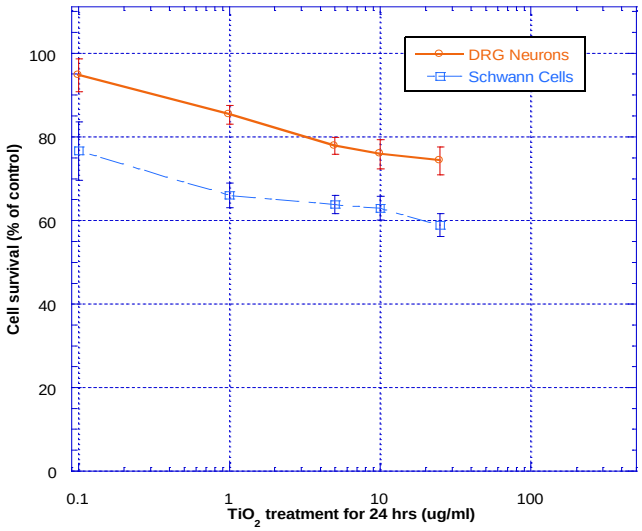

Figure 3: Effects of TiO_2 nanoparticles on DRG neurons or Schwann cells. Values are mean ± SEM of 12 determinations and replicate experiments show the same trend. DRG neurons and Schwann cells treated with TiO_2 nanoparticles for 24 hours are marked red and blue, respectively.

Results of our present and ongoing studies have confirmed that neural cells of the peripheral nervous system, similar to their counter parts in the central nervous system, are also susceptible to the cytotoxicity of SiO_2 and TiO_2 nanoparticles. Thus, these results are consistent with our hypothesis that our non-contact co-culture model of Schwann cells and DRG neurons can be useful to study toxic effects of various nanoparticles on peripheral nervous system.

The results of this and our ongoing studies suggest that nanoparticles may exert differential cytotoxic effects on neural cell types of the peripheral nervous system. As such, our results may assume pathophysiological importance in the environmental health impact of nanoparticles. Obviously, it is an important area that deserves further study.

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