Co-Culturing Dorsal Root Ganglion Neurons with Schwann Cells Protects Them against the Cytotoxic Effects of Silver Nanoparticles

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ABSTRACT

Because of their presumed inertness, silver nanoparticles are increasingly employed in many consumer products. Nonetheless, their environmental and health hazard impact are not understood. Our previous studies led us to develop a co-culture model consisting of dorsal root ganglion (DRG) neurons and Schwann cells and to employ it to investigate our hypothesis that co-culturing DRG neurons with Schwann cells imparts protection on them against cytotoxicity induced by silver nanoparticles. Our results indicated that silver nanoparticles induced concentration- and time-related decreases in survival of DRG neurons or Schwann cells in monotypic cultures: both cell types withstood the cytotoxicity of silver nanoparticles and survived better when maintained in co-cultures. DRG neurons and co-cultures of DRG neurons and Schwann cells, but not Schwann cells alone, expressed synapsin. Silver nanoparticles suppressed synapsin expression in DRG neurons alone but not in co-cultures with Schwann cells. Schwann cells and co-cultures of DRG neurons and Schwann cells, but not DRG neurons alone, expressed glial fibrillary acidic protein (GFAP). However, silver nanoparticles markedly suppressed GFAP expression in Schwann cells alone but not in co-cultures with DRG neurons. Thus, our results provide support for our hypothesis and may be relevant to toxicological studies prior to clinical trials of drugs formulated with agents containing silver nanoparticles.

Keywords: dorsal root ganglion neurons, Schwann cells, co-culture, silver nanoparticles, cytotoxicity, nanotoxicity

1 INTRODUCTION

Recently many nanoparticles have been gainfully exploited in a variety of applications in diverse industries including biotechnology (e.g., as probes in cell & tissue imaging and in new drug delivery & targeting) because of their unique physico-chemical properties [1, 2]. Because of their presumed inertness, silver nanoparticles are increasing employed in many consumer products and are thus produced on an industrial and large scale [3-5]. Nonetheless, their environmental and health hazard impact are unknown [3-5].

Over the last decade, we have been developing a variety of neural and non-neural cell types as *in vitro* models for systematic investigation of putative cytotoxicity of various nanomaterials, including metallic and non-metallic nanoparticles [6-11]. More recently, we have developed two non-tumor neural cell models *in vitro* for systematic investigation of putative cytotoxicity of various nanomaterials including nanoparticles employing dorsal root ganglion (DRG) neurons and Schwann cells, which are physiologically important neural cell types of the peripheral nervous system [12-13].

Our previous findings that several metallic nanoparticles exerted cytotoxic effects on DRG neurons and Schwann cells [12-13] strongly suggest that they constitute excellent non-tumor neural models for elucidating the cellular and molecular mechanisms underlying the cytotoxicity of nanoparticles and other nanomaterials in neural cells and such mechanistic studies may have pathophysiological and/or pathogenic implications in neurodegeneration and nerve degeneration. Thus, our previous studies led us to develop a co-culture model consisting of DRG neurons and

Schwann cells and to employ it to investigate the hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against the cytotoxicity induced by silver nanoparticles.

2 MATERIALS AND METHODS

2.1 Materials

S16 Schwann cells were obtained from ATCC (Manassas,VA, USA). DRG (50B11) neurons were kind gifts from Dr Höke's Laboratory at Johns Hopkins School of Medicine. Thiazolyl blue tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Silver nitrate ($AgNO_3$) and trisodium citrate ($C_6H_5Na_3O_7•2H_2O$) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The monoclonal antibody against glial fibrillary acidic protein (GFAP) was obtained from Cell Signaling Technology (Beverly, MA, USA). The polyoclonal antibody against synapsin, goat anti-rabbit IgG secondary antibody, and goat anti-mouse IgG secondary antibody were purchased from Abcam Inc. (Cambridge, MA, USA). The monoclonal antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All chemicals were of analytical grade and were usually obtained from Sigma-Aldrich.

2.2 Cell Culture

S16 Schwann cells or DRG neurons were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) FBS and 25 mM glucose at 37˚C and with 5% (v/v) CO_2.

2.3 Cell viability assay

Cell survival/proliferation was determined by using the modified MTT assay [8-11]. S16 Schwann cells were seeded (3000 cells/well) into a 24-well plate and allowed to attach and grow for 1 hour. Then the same number of DRG neurons were seeded onto the substratum layer of Schwann cells and cultured as described above. After 1 hour, cells were treated with or without specified concentrations of silver nanoparticles which were prepared as described previously [11]. Monotypic cultures (i.e., Schwann cells or DRG neurons alone) were also set up similarly. The plates so prepared were incubated for 1, 2, 3, 4, or 5 days at 37˚C. At the end of the incubation period, 100 μL of 5 mg/mL MTT dye in PBS was added into each well and the plates were incubated for an additional 4 hours at 37˚C. The purple-colored insoluble formazan crystals in viable cells were dissolved using DMSO and the subsequent absorbance (designated as X) of the content of each well was measured at 570 nm using a Bio-Tek Synergy HT Plate Reader (Winooski, VT, USA) [14].

The medium and silver nanoparticles by themselves had absorbance: thus, their absorbance (i.e., the control sets of wells) had to be subtracted from the absorbance of live cells with or without different concentrations of silver nanoparticles treatments. The control sets of wells were set up alongside those sets of wells in the plates as detailed in the preceding paragraph except that the control sets of wells did not contain any seeded cells. At the end of the specified culture period, 100 μL of 5 mg/mL MTT dye in PBS was added into each well and the plates were incubated for an additional 4 hours at 37˚C. The subsequent absorbance (designated as Y) of the content of each well was measured at 570 nm as described above. (X-Y) was taken as the absorbance attributed to viable cells in each well. The absorbance of co-cultures of DRG neurons and Schwann cells were compared with the sum of absorbance of monotypic cultures (i.e., Schwann cells or DRG neurons alone).

2.4 Western blot analysis

Expression of synapsin and GFAP was determined by Western blot analysis [10, 15]. Cells treated with or without silver nanoparticles (125 μL) were collected and homogenized. Protein content of the homogenates dissolved by 10 M NaOH for 3 days was then determined using the bicinchoninic acid technique as described previously [10, 15]. Equal amounts of protein from the samples were loaded onto the lanes of the gels, and the proteins were separated by polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. Monoclonal or polyclonal antibodies against the respective proteins were then used to probe the proteins of interest. The polyvinylidene fluoride membrane containing the target protein was then developed using the chemiluminescence technique on an X-ray film to assess the extent of expression of respective proteins [10, 15].

2.5 Statistical analysis of data

Experiments were performed at least three times with a minimum of 6 replicates for each set, and all data were recorded as mean ± standard deviation.

3 RESULTS AND DISCUSSION

Employing the modified MTT assay, we studied the effects of different concentrations of silver nanoparticles on survival, proliferation, and/or growth of the Schwann cells alone, DRG neurons alone, and DRG neurons co-cultured with Schwann cells. As shown in Figures 1, 2 and 3, the survival/proliferation of cells (both DRG neurons and Schwann cells) in co-cultures was better than that of cells in monotypic cultures after 4 days' culture (compare the dark line with the green line in Figures 1, 2, and 3). Silver

nanoparticles were cytotoxic to DRG neurons and Schwann cells. At treatment concentrations of 25 µL and higher, silver nanoparticles induced concentration- and time-related decreases in survival of DRG neurons or Schwann cells in monotypic cultures. However, both cell types survived better in co-cultures when they were exposed to higher concentrations (75 µL and 125 µL) of silver nanoparticles compare to when they were exposed to the silver nanoparticles in monotypic cultures (compare the orange line with the brown line in Figures 2 and 3), especially at the concentration of 125 µL (compare Figures 2 and 3). Thus, we employed the concentration of 125 µL to do all subsequent experiments. All these results (Figure 1-3) indicated that co-culturing DRG neurons with Schwann cells imparted some protection on them against the cytotoxicity induced by silver nanoparticles.

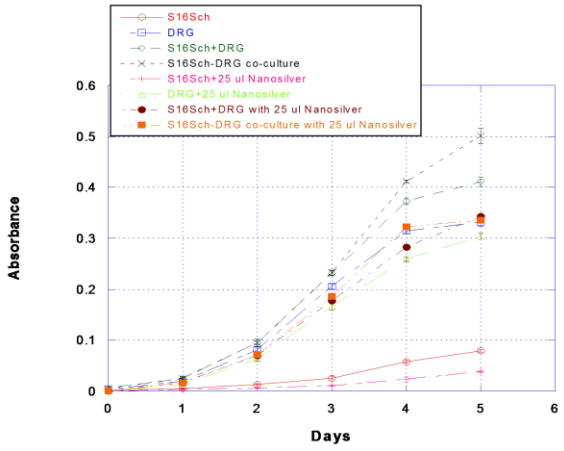

Figure 1: Effects of treatment with 25 µL silver nanoparticles for 5 days on the survival/proliferation of S16 Schwann cells alone, DRG neurons alone, or S16 Schwann cells co-cultured with DRG neurons.

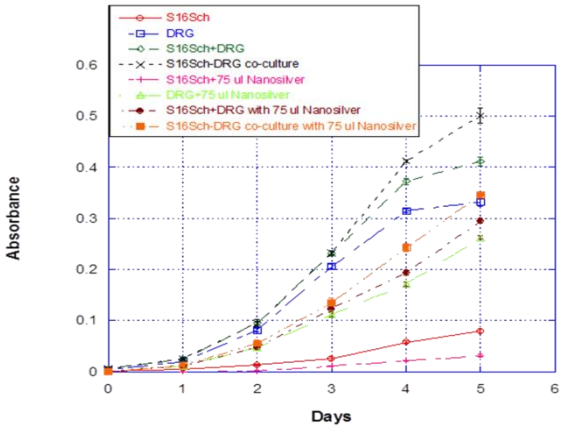

Figure 2: Effects of treatment with 75 µL silver nanoparticles for 5 days on the survival/proliferation of S16 Schwann cells alone, DRG neurons alone, or S16 Schwann cells co-cultured with DRG neurons.

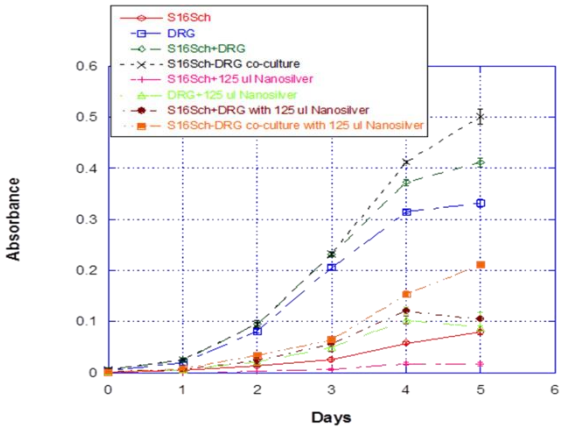

Figure 3: Effects of treatment with 125 µL silver nanoparticles for 5 days on the survival/proliferation of S16 Schwann cells alone, DRG neurons alone, or S16 Schwann cells co-cultured with DRG neurons.

To determine some of the cellular and molecular mechanisms underlying this phenomenon of "protection" in co-cultures, we examined the expression of two cellular biomarkers, namely synapsin (a marker of neuronal and synaptic function) and GFAP (a glial marker), in DRG neurons and Schwann cells either singly in monotypic cultures or in co-cultures in the presence or absence of silver nanoparticles at the concentration of 125 µL by Western blot analysis (data not shown). As expected of a neuronal biomarker, DRG neurons, but not Schwann cells, expressed synapsin. Similarly, co-cultures of DRG neurons and Schwann cells also expressed synapsin. By contrast, synapsin expression was increased in DRG neurons when they were co-cultured with Schwann cells and treated with or without silver nanoparticles, suggesting that co-culturing with Schwann cells exerted beneficial effects on DRG neurons and protect them from the cytotoxicity of silver nanoparticles. As expected of a glial marker, Schwann cells, but not DRG neurons, expressed GFAP. Similarly co-cultures of DRG neurons and Schwann cells also expressed GFAP. However, the presence of silver nanoparticles markedly suppressed GFAP expression in Schwann cells in monotypic culture but not in co-cultures of DRG neurons with Schwann cells, indicating that co-culturing with DRG neurons appeared to render the Schwann cells less susceptible to the cytotoxicity of silver nanoparticles. These results are consistent with our hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against the cytotoxicity induced by silver nanoparticles.

4 CONCLUSIONS

Results of our ongoing studies revealed that, while silver nanoparticles induced concentration- and time-related

decreases in survival of DRG neurons or Schwann cells in monotypic cultures, both cell types appeared to withstand the cytotoxicity of silver nanoparticles and survive better when maintained in co-cultures. Synapsin expression was increased in DRG neurons when they were co-cultured with Schwann cells and treated with or without silver nanoparticles. GFAP expression was increased in Schwann cells when they were co-cultured with DRG neurons and treated with silver nanoparticles. Taken together, our results are consistent with our hypothesis that co-culturing DRG neurons with Schwann cells imparts some protection on them against the cytotoxicity induced by silver nanoparticles and may have pathophysiological implications in the biocompatibility and health hazard of silver nanoparticles. Moreover, our findings may be of relevance to toxicological studies of drugs formulated with agents containing silver nanoparticles before such drugs can be considered for human clinical trials.

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