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Full paper

Quantitative analysis of intraneuronal transport in human iPS neurons

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ABSTRACT

Induced pluripotent stem (iPS) cells are promising tools to investigate disease mechanism and develop new drugs. Intraneuronal transport, which is fundamental for neuronal survival and function, is vulnerable to various pharmacological and chemical agents and is disrupted in some neurodegenerative disorders. We applied a quantification method for axonal transport by counting CM-DiI–labeled particles traveling along the neurite, which allowed us to monitor and quantitate, for the first time, intraneuronal transport in human neurons differentiated from iPS cells (iCell neurons). We evaluated the acute effects of several anti-neoplastic agents that have been previously shown to affect intraneuronal transport. Vincristine, paclitaxel and oxaliplatin decreased the number of moving particle along neurites. Cisplatin, however, produced no effect on intraneuronal transport, which is in contrast to our previous report indicating that it inhibits transport in chick dorsal root ganglion neurons. Our system may be a useful method for assessing intraneuronal transport and neurotoxicity in human iPS neurons.

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1. Introduction

Intraneuronal transport is fundamental for neuronal survival, morphogenesis, and homeostatic functions (1–4). Many proteins, mRNAs, and organelles are selectively carried to their final destinations, either axons or dendrites, within neurons. This movement along microtubules is powered by the molecular motors of the kinesins and dynein super families. Recently, signal-dependent regulation of intracellular transport has drawn attention (5). For instance, retrograde axonal transport mediates the biological effect of the axon guidance molecule semaphorin3A and regulates dendritic localization of AMPA-type glutamate receptors and dendritic patterning (4). These findings illustrate the importance of intracellular transport in regulating higher functions of the nervous system. Disturbances in axonal transport and accumulations of organelles and proteins in axons, dendrites, and/or cell bodies contribute to the pathogenesis of many human neurodegenerative diseases (6–10). In addition, disruption of intraneuronal transport has been implicated in neuropathy induced by anti-neoplastic agents (11, 12). Thus, a simple, accurate, and objective evaluating system for intraneuronal transport is needed.

We previously developed a computer-assisted system for quantifying axonal transport by counting moving organelles labeled with chloromethylbenzamide dialkycarbocyanine (CM-DiI), a lipophilic dye, along the axons of chick dorsal root ganglion (DRG) neurons (1). The aim of the current study was to establish a system to monitor intraneuronal transport in human neurons differentiated from induced pluripotent stem (iPS) cells by employing iCell neurons, which are now commercially available (13–15). For this purpose, we characterized the intracellular transport of iCell neurons and evaluated how it was affected by anti-neoplastic agents that are known to exert neurotoxicity by affecting axonal transport (1, 11, 12). Our system may be a useful tool for assessing neurotoxicity based on transport in human iPS neurons.

2. Materials and methods

2.1. Materials

CM-DiI was purchased from Invitrogen (Eugene, OR, USA). Poly-D-lysine hydrobromide, laminin, vincristine sulfate salt, 5-

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fluorouracil (5-FU), *cis*-diammineplatinum dichloride (cisplatin), and oxaliplatin were from Sigma (St. Louis, MO, USA). Paclitaxel and methotrexate were from Wako Pure Chemicals (Osaka, Japan).

2.2. Culture of human iPS neurons

iCell neurons were provided from Cellular Dynamics International (CDI, Madison, WI, USA). The human iPS cells used to generate iCell Products were independently derived for research use from a well-characterized, commercially available human fibroblast cell population originally isolated under the guidance of the National Institute on Aging and the National Institutes of Health. The cells were obtained under the informed consent practices in place at that time. iCell neurons were plated at concentrations of 5.4×10^4 cells per dish on 35-mm cell culture glass-based dishes (Iwaki, Tokyo, Japan) pre-coated with poly-D-lysine (50 μg/ml) and then with laminin (3.3 μg/ml) in water. iCell neurons were cultured in the iCell Neurons Maintenance Medium supplemented with iCell Neurons Medium Supplement (CDI). The culture medium exchange was performed 24 h after plating and was repeated every 2–3 days until the start of the experiment. iCell neurons were fixed for immunostaining or loaded with CM-DiI at 5–16 days *in vitro* (DIV) for monitoring intraneuronal transport.

2.3. Immunocytochemistry

Immunocytochemistry was done by a standard protocol under permeabilization with 0.1% TritonX-100. The antibodies used for the staining of cultured iCell neurons were as follows: anti-Tau1 mouse monoclonal antibody (MAB3420, 1:500 dilution, Millipore, Billerica, MA, USA) and anti-MAP2 rabbit polyclonal antibody (PRB-547C, 1:500 dilution, Covance, Princeton, NJ, USA). Immunostained neurons were analyzed by laser-scanning microscope (LSM510) with a water-immersed objective lens at × 40 (C-Apochromat/1.2 W corr) equipped with an Axioplan 2 imaging microscope (Carl Zeiss, Göttingen, Germany).

2.4. Live imaging and application of anti-neoplastic agents

iCell neurons stained with CM-DiI were observed by confocal microscopy. To visualize the moving particles, neurons were first cultured for 30 min in the medium containing CM-DiI (1 μM), washed three times in Leibovitz's L-15 medium (L-15 medium) (Invitrogen, Auckland, NZ), and then incubated for 4 h in CM-DiI-free culture medium to remove any excess dye loaded in the iCell neurons. CM-DiI-labeled moving particles along the neurites were observed by a laser-scanning microscope (LSM5 PASCAL) equipped with an Axioplan 2 imaging microscope (Carl Zeiss) with a water-immersed objective set at × 40 (C-apochromat/1.2 W corr). The images were typically 644.00 by 130.00 pixels, corresponding to an image size of 75.00 by 15.10 μm with a pixel size of 0.116 μm (~0.12 μm). The images were collected every 500.00 ms, at intervals of 0.25 μs, for 2.00 min. For quantification of the motility of the particles, the 240 images captured during 2 min were analyzed by computer-assisted software. To minimize data fluctuation, we estimated the total number of particles, defined as the summation of the data obtained at 74 points at 1.00-μm intervals along the length of the neurite starting from the origin of the neurite (1). Particles ranging in diameter size from 0.93 to 1.86 μm diameter were extracted for the estimation in this system. This particle size is consistent with that of vesicles containing GAP-43-GFP ranging from 0.2 to 4.2 μm (16). Electron microscopic analysis of the structure of the axoplasm by the method of quick-freezing and deep-etching revealed that the size of mitochondria or lysosome is within the range of 0.5–2 μm (17). Based on this vesicular size, it is unlikely that the vesicles consisted of several small independent vesicles. This assay system requires imaging transport within neurites that are longer than 70 μm. The velocity histogram of the intraneuronal transport was obtained from the quantitative analysis using this software. To investigate the effects of anti-neoplastic agents, iCell neurons were incubated for 24 h in the presence or absence of the agents, and the intraneuronal transport of CM-DiI–labeled particles was then monitored as described above.

2.5. Cytotoxicity of CM-DiI on chick DRG neurons

After 24-h incubation in the presence or absence of CM-DiI (1 μM), cell viability was assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (18).

2.6. Statistical analysis

Two-tailed Student *t*-test was applied to the data as indicated in the figure legends. Differences were considered to be significant at $P < 0.05$. All data in the text and figures are presented as means ± SEM.

3. Results

3.1. Characterization of iCell neurons

iCell neurons generated through a proprietary forebrain differentiation protocol represent a pure neuronal population, mainly of forebrain identity, consisting of a mixed proportion of glutamatergic and GABAergic neurons (19). Functional studies showed that these neurons express ionotropic glutamate receptors, gamma-aminobutyric acid type A receptors, voltage-gated sodium and calcium channels (14). To further characterize the neurons at different stages of culture, immunocytochemistry was done at 5 and 19 DIV. Neurites stained equally with antibodies against MAP2 and Tau1, which are dendrite and axon marker proteins, respectively (Fig. 1). The equal staining suggests that iCell neurons did not acquire their axon-dendrite polarity during *in vitro* developmental stages. Meanwhile, Nomarski images showed a subtle morphological difference of the neurons at these stages of culture: neurons at 16 DIV generally had pyramidal-like cell bodies with neurites, while those at 5 DIV had spindle-shaped cell bodies with long bipolar projections (Fig. 2A and B). Nevertheless, there were no differences in the mean number of neurites per cell (3.20 ± 0.35 at 5 DIV, 3.50 ± 0.26 at 16 DIV) or in the length of neurites (55.15 ± 6.38 μm at 5 DIV, 54.16 ± 5.33 μm at 16 DIV) ($n = 10$) (Fig. 2A and B).

3.2. Visualization of CM-DiI-labeled moving particles along the neurites

After the destaining procedure, approximately one third of the neurons per culture dish were still positive with CM-DiI. In these neurons, CM-DiI–labeled particles that were moving were clearly visualized along neurites under confocal microscopy (Fig. 2C and D). There were no morphological differences between CM-DiI–positive and CM-DiI–negative neurons at 5 and 16 DIV (Fig. 2A and B). For example, the mean number of neurites per cell and the length of neurites in CM-DiI–positive and CM-DiI–negative neurons were 3.41 ± 0.33 and 55.19 ± 5.50 μm ($n = 12$) and 3.41 ± 0.31 and 52.69 ± 4.79 μm ($n = 12$), respectively.

We estimated that the number of CM-DiI–labeled particles moving in both anterograde and retrograde directions was 12–13 particles/min, which was lower compared to the number of moving particles detected by differential interference contrast

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